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Anaerobic Treatment of Wastewaters Containing Perchlorate from Munitions Handling and Production

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Anaerobic Treatment of Wastewaters from Munitions Handling/Production Containing Perchlorate

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Abstract: Perchlorate, an oxidizer routinely used in solid rocket motors, is easily washed out of old motors. The washout operation, however, leads to wastewater containing perchlorate. Royal Demolition Explosive (RDX), a major component of military high explosives, is transferred to Army industrial wastewaters during assembly of new munitions and during demilitarization of old munitions. New high explosives contain perchlorate, commingling perchlorate and RDX for the first time. In addition to their damaging effect to the environment, perchlorate and RDX can also be detrimental to human health.

This study focused on determining the effectiveness of removing perchlorate and RDX individually and when commingled, using ethanol as an electron donor at steady-state conditions. Three laboratory-scale anaerobic fluidized bed reactors were monitored. A fourth reactor, located at McAlester Army Ammunition Plant, currently used to treat pinkwater, was also tested.

The experimental results demonstrated that the biodegradation of perchlorate and RDX was more effective in bioreactors receiving a single contaminant than in the bioreactor fed both contaminants. However, the results also show that perchlorate and RDX can be treated in a single reactor to levels below discharge limitations. Results from perturbation experiments revealed that competition within the microbial consortia for substrate played a major role in determining the structure of that community.

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Preface

This study was conducted in part for Headquarters, Department of the Army, under Program Element 622720, “Environmental Quality Technology,” Project D048, Work Unit 118997, “Industrial Activities Readiness,” Activity Title “Anaerobic GAC-FBR for Perchlorate Treatment,” and in part for the Joint Munitions Command (JMC) through reimbursable funding. The JMC point of contact is Frank Novak. The technical reviewer was Dr. Paul Hatzinger of Shaw Environmental, Lawrenceville, NJ.

The work was managed and executed by the Environmental Processes Branch (CN-E) of the Installations Division (CN), Construction Engineering Research Laboratory (CERL). The CERL principal investigator was Dr. Stephen W. Maloney. Part of this work was done at the University of Cincinnati by co-authors Dr. Makram T. Suidan and Emina Atikovic, and part of the work was done at McAlester Army Ammunition Plant with the assistance of Ryan Williams (Environmental Office), Bob Branam (Chief of Water and Wastewater Utilities), and Bradley Black (Analytical Chemistry). Debbie Curtin is Chief, CN-E, and Dr. John T. Bandy is Chief, CN. Martin J. Savoie is Technical Director for the Installations business area. The Deputy Director of CERL is Dr. Kirankumar V. Topudurti, and the Director is Dr. Ilker R. Adiguzel.

CERL is an element of the U.S. Army Engineer Research and Development Center (ERDC), U.S. Army Corps of Engineers. The Commander and Executive Director of ERDC is COL Richard B. Jenkins, and the Director of ERDC is Dr. James R. Houston.

Unit Conversion Factors

Multiply	By	To Obtain
feet	0.3048	meters
gallons (U.S. liquid)	3.785412 E-03	cubic meters
inches	0.0254	meters

1 Introduction

Background

Perchlorate is an oxidant that is widely used in propellants such as solid rocket motors, and in commercial fireworks and road flares. It has recently been put into new munitions formulations as an oxidizer. Although it is not yet in any production-scale munitions, it is being test packed into new munitions. A U.S. Air Force formulation pairs perchlorate with Royal Demolition Explosive (RDX), and numerous proposed Army formulations use it in conjunction with RDX as well as other new energetic chemicals.

Unlike most high explosive compounds in use today, perchlorate is an ionic species that dissolves readily in water. The most common method for removing energetic compounds from wastewaters generated during munitions handling is adsorption onto granular activated carbon (GAC). This method is used because the most common existing high explosives such as trinitrotoluene (TNT) and RDX are only sparingly soluble in water, and their non-polar nature allows GAC to have a fairly large capacity for removing them from water via adsorption. The highly polar perchlorate would not be attracted to the GAC's surface, and would be expected to pass through the GAC with only negligible retention.

The introduction of perchlorate in the high explosive inventory requires the development of a method to treat wastewaters generated during the production of the munitions. McAlester Army Ammunition Plant (MCAAP, McAlester, OK) generated small amounts of perchlorate contaminated munitions wastewater during the test packing of an Air Force bomb, and was able to dispose of it during the demonstration study described herein, funded by the Joint Munitions Command (JMC). Holston AAP in Tennessee has also generated perchlorate-contaminated wastewater during test packing of munitions containing PAX-21 (an Army explosive developed by the Armaments Research, Development and Engineering Center, Picatinny Arsenal, NJ). They placed the wastewater in 55-gallon drums and disposed of the drums in a hazardous waste landfill.

Objectives

The objective of this project was to develop a method to treat perchlorate while simultaneously treating RDX and TNT. The laboratory-scale work

focusing on RDX was done at the University of Cincinnati, with two main objectives:

1. Determine the treatability of perchlorate and RDX by anaerobic bacteria, alone and in combination, to evaluate whether the presence of one compound interferes with treatment of the other, and
2. Determine the effects of sporadic episodes of perchlorate on the treatment process, after the treatment process had been demonstrated and had reached steady-state removal.

The research focusing on treatment of perchlorate in an existing biological process developed for pinkwater (wastewater containing TNT and RDX) was conducted at MCAAP. This study's third main objective is therefore summarized as:

3. Determine the effect of short-term perchlorate discharges to an anaerobic biological treatment process already in place, operating on actual pinkwater from a production facility.

Approach

This study was conducted simultaneously on the laboratory- and pilot-scale. Experiments were designed and executed to evaluate the potential competition between perchlorate and RDX under highly controlled laboratory-scale conditions. At the same time, short duration tests were conducted at MCAAP by injecting a highly concentrated perchlorate solution into the pinkwater feed line of an existing treatment facility designed for pinkwater (industrial wastewater containing TNT, RDX, and their by-products as generated in the load assemble and pack, or washout of conventional munitions).

Mode of technology transfer

The results of the steady-state part this study have been presented at a Water Environment Federation conference, and further publications and presentations will be made at future conferences. ERDC is also working directly with MCAAP in development of cost and technical data needed to install this technology at full scale. ERDC/CERL will work with the Army Environmental Command (AEC) to develop technology transfer options at other installations as they begin to introduce commingled explosives and perchlorate in their wastewater.

As part of the technology transfer, ERDC/CERL has made presentations to representatives of the JMC and the Oklahoma Water Board, to gain acceptance of the technology by stakeholders and regulators.

2 Literature Review

Perchlorate (ClO_4^-)

Production and handling of a new high explosive mixture generates wastewater contaminated with energetic compounds such as perchlorate and RDX. A major source of perchlorate contamination comes from ammonium perchlorate, which is used as the oxidizer component and primary ingredient in solid propellants for rockets, missiles, and fireworks. In addition, the perchlorate anion can be introduced to the environment in other forms of salts such as sodium, potassium, or magnesium perchlorate. Perchlorate is an oxidizer that has been routinely used in solid rocket motors by the Department of Defense (DoD) and National Aeronautics and Space Administration.

One of the advantages of perchlorate is that it is easily washed out of old rocket motors and can be crystallized for reuse in civilian applications such as commercial fireworks and road flares. Perchlorate is an anion, which consists of a central chlorine atom surrounded by four oxygen atoms. In this tetrahedral conformation, negative charge is evenly distributed among four oxygen atoms, weakening the ability of any positive metallic centers or common reductants to directly attack the chlorine. Due to this feeble reactivity and high water solubility, perchlorate ions can persist in the environment for decades (Urbansky 2000). This makes the treatment of perchlorate contamination in the aqueous environment rather challenging.

Contamination and the human health threat

In 1985 perchlorate contamination was detected by the U.S. Environmental Protection Agency (EPA) in groundwater wells of the San Gabriel Valley, near Los Angeles, CA, with concentrations ranging from 110–2600 $\mu\text{g/L}$ (Motzer 2001). This contaminant was not regulated until 1997, when a provisional action level of 18 $\mu\text{g/L}$ was imposed (Coates and Achenbach 2004). In 1998 perchlorate was added to the EPA contaminant candidate list for drinking water supplies, and in 2002, a revised provisional action level of 1 $\mu\text{g/L}$ was recommended (Coates and Achenbach 2004 ; Coleman et al. 2003). Massachusetts recently set a regulatory limit at 2 $\mu\text{g/L}$. Full copies of the regulation and the response-to-comment document are available on the Massachusetts Department of Environmental Protection

web site at: <http://mass.gov/dep/water/drinking/percinfo.htm>.

California has established a Public Health Goal of 6 µg/L

(<http://www.oehha.org/water/phg/pdf/perchlorate3docs.pdf>).

Besides being very damaging to the environment, perchlorate contamination also represents a direct threat to human health (Song and Logan 2004). Due to its ability to competitively inhibit the transport of iodine from blood into the thyroid gland, perchlorate is considered to be an endocrine disrupting compound (Urbansky 2000). In addition, prolonged deficiency in iodine uptake can result in hypothyroidism, mental retardation, and speech and hearing degradation.

Treatment technology

Perchlorate treatment technologies are generally classified into two categories, destructive technologies or removal technologies. The destructive processes can be classified into two subcategories, biological and chemical treatments. The nondestructive removal processes are mainly concentrated on ion exchange resins. To date, biological reduction of perchlorate appears to hold the most promise for the treatment of large flows of perchlorate-laden waters (Urbansky and Schock 1999). Industrial wastewater containing perchlorate generated from demilitarization of large rocket motors has been effectively treated using anaerobic bacteria in a suspended growth biological system (ESTCP 2000). Groundwater contaminated by perchlorate was shown to be treatable using biomass attached in GAC in fluidized bed reactors (FBRs; Polk et al. 2002 and Hatzinger 2005). Perchlorate commingled with RDX was shown to be treatable using FBRs containing GAC (Veenstra et al. 2003; Fuller et al. 2007), but it was not clear whether the RDX was removed by bioactivity or adsorption. The commingled study was performed on groundwater where the concentrations of the contaminants were less than 1 mg/L. Mass balances based on influent and effluent concentrations, and an undefined extraction procedure for adsorbed RDX (Fuller et al. 2007), were used to postulate extent of biodegradation, but the presence of GAC remained a complicating factor. Nevertheless, there is no common biological treatment for industrial wastewater containing perchlorate and explosives together and at high concentrations, as this wastewater results from a new munition formulation.

Royal Demolition Explosive

RDX, also known as hexahydro-1,2,5-trinitro-1,3,5-triazine, is a major component of military high explosives used in a large variety of munitions. It is a white solid made of orthorhombic colorless crystals that are soluble in certain organic solvents but not in water. These crystals are very stable chemically and thermally (De Luca et al. 1999). Due to its high stability, RDX is widely used in various military and civilian applications. It is transferred to Army industrial wastewaters during the load, assemble, and pack operations for new munitions, and during hot water or steam washout for disposal and deactivation of old munitions (commonly referred to as demilitarization). Manufacture, use, and destruction of explosive compounds can lead to leakage of highly toxic material into the environment (Van Aken et al. 2004). Because of its harmful effects, RDX was placed on EPA's priority pollutant list (Van Aken et al. 2004). Furthermore, toxicity of RDX is shown to be detrimental to human health as well as to other classes of organisms such as algae, plants, earthworms, aquatic invertebrates, and animals including mammals (Van Aken et al. 2004).

Treatment technology

In the past, treatment of RDX wastes involved filling lagoons with the contaminated water and letting that water evaporate (Maloney et al. 2002). This resulted in introduction of RDX into the soil and ground water. Since this process proved to be harmful to the environment, other treatment technologies such as biodegradation and adsorption have been attempted (Van Aken et al. 2004). Biodegradation of RDX can occur under anaerobic, methanogenic, sulfogenic, and nitrate-reducing conditions (Hawari et al. 2000). However, the most common method for RDX treatment today is adsorption on GAC.

Biodegradation pathway

Earlier work done by McCormick et al. (1981) proposed that biodegradation of RDX proceeds under anaerobic conditions via successive reduction of the nitro groups, to the point of destabilization and fragmentation of the ring. In this pathway, RDX is sequentially reduced to the nitroso derivatives hexahydro-1-nitroso-3, 5-dinitro-1, 3,5-triazine (MNX), hexahydro-1, 3,5-trinitroso-5-nitro-1, 3,5-triazine (DNX), and hexahydro-1, 3,5-trinitroso-1, 3,5-triazine (TNX) yielding formaldehyde and methanol as end products (McCormick et al. 1981). Methylene dinitramine has also

been shown to be an intermediate in RDX biodegradation (Halasz et al. 2002) under certain conditions.

Anaerobic fluidized bed reactors

The use of anaerobic fluidized bed reactors (AFBRs) is well established for the treatment of industrial and difficult to biodegrade wastewaters (Grace and Bi 1997). This process entails the passage of a fluid through a bed of particulate solids, supported over a fluid-distributing plate, at a flow rate above a minimum critical value, causing the solids to behave like a liquid. The medium particles serve to support the attachment of biomass. The performance of the AFBR is often compared with that of more conventional suspended growth and fixed film biological reactors. The results invariably show superior performance of the AFBRs. Some of the advantages of using this treatment process follow (Gupta and Sathiyamoorthy 1999):

- Large solids-liquid interfacial area
- Low washout of microbes from the system
- Biomass recovery at high substrate loading
- Minimal sludge recycle, and
- Clog-free operation while biomass accumulates.

In addition, fluidization can also reduce the thickness of the concentration boundary layer leading to decreased resistance to mass transport (Suidan et al. 1988). Wang et al. (1987) demonstrated that these characteristics of the AFBRs result in a cost-effective biological treatment process for low-strength wastewater.

Anaerobic biological treatment of pinkwater

Pinkwater is a common term used by DoD industrial personnel to refer to wastewater that is contaminated with TNT and RDX. This wastewater is generated as the TNT and RDX are melted to pour into munitions, when old munitions are decommissioned by removing the high explosives, and during washdown operations after either of the first two. Although TNT and RDX are the main hazardous components, washdown operations can lead to a variety of other contaminants in the wastewater. The U.S. Army Engineer Research and Development Center's Construction Engineering Research Laboratory (ERDC/CERL) has demonstrated an anaerobic treatment technology using GAC as the bacteria attachment media in FBRs (Maloney et al. 2002; ESTCP 2003).

Denaturing gradient gel electrophoresis

To better understand the biodegradation process of perchlorate and RDX in fluidized bed reactors, it is important to understand the structure, function, and dynamics of microbial communities. In order to follow the changes in the microbial community, some molecular fingerprinting techniques have to be used. These techniques give insight on the presence and relative abundance of different microbial populations. Genetic fingerprinting techniques are rapid and easy to use. Due to their ability for multiple sample analysis, these methods allow comparison of genetic diversity between microbial communities (Muyzer 1999). Muyzer et al. (1993) reported a new fingerprinting technique to describe microbial population diversity in which they introduced denaturing gradient gel electrophoresis (DGGE). DGGE allows deoxyribonucleic acid (DNA) fragments of the same length, but different base sequence, to be separated.

Separation of the DNA fragments is based on the decreased electrophoretic mobility of partially melted fragments in the polyacrylamide gels with the linearly increasing DNA denaturant, usually urea and formamide (Muyzer et al. 2004). DNA migrates in so-called “melting domains,” which are the stretches of base pairs (bp) with the identical melting temperatures. Once a melting domain reaches its melting temperature, the DNA double helix will partially unwind and the migration will stop. Variation in melting temperatures, due to the difference in bp sequence of melting domains, will stop the migration of DNA fragments at different positions in the gel; therefore, allowing for better separation.

Using the DGGE technique approximately 50% of the sequence variation can be detected (up to 500 bp). In order to increase this percentage to 100%, a 40- to 50-bp GC-clamp (guanine-cytosine rich sequence) is added to the 5' end of one of the polymerase chain reaction (PCR) primers (Muyzer et al. 2004). This GC-clamp is co-amplified and introduced into the DNA fragments where it acts as a high melting domain, preventing the complete dissociation of the double helix into single strands. The DGGE bands can be visualized using ethidium bromide stain, silver stain, or SYBR Green I. SYBR Green I is most widely used due to its ability to minimize background staining, allowing for visualization of even less dominant bands (Muyzer et al. 1998).

DGGE advantages

DGGE can provide the characterization of community composition and diversity as well as shifts in population. The advantage of DGGE is that many samples collected at different time intervals can be analyzed simultaneously, which makes DGGE a powerful tool for monitoring microbial response to perturbations. In addition, the bands can be excised from the gel and sequenced, which allows for determination of phylogenetic relationship of microbial communities (Muyzer 1999).

DGGE disadvantages

The disadvantages of DGGE pertain to the fact that only relatively short DNA fragments (500 bp) can be separated. Due to the sequence variation, poor separation of DNA fragments can also occur.

3 Experimental Design and Materials

AFBR operational parameters

Three 9.1-L AFBRs were used for the laboratory-scale study (Figure 1). The first bioreactor (P) was used to evaluate the effectiveness of reducing perchlorate with ethanol as an electron donor. The second bioreactor (R) was used to determine the reduction of RDX under the same electron donor conditions, while the third bioreactor (P&R) was set up to examine the co-treatment of both perchlorate and RDX. Each bioreactor consisted of a jacketed main column, and influent and effluent headers. The inner tube (96.5 cm long, 10.2 cm inner diameter) was constructed of Plexiglas and was enclosed in an outer jacket also constructed from a Plexiglas tube. Water was circulated through the annular space between the two tubes of the AFBRs from a constant temperature bath (Model 28M-L Isotemp Water Bath, Fisher Scientific, Pittsburgh, PA) to maintain a constant temperature of 35 °C within the columns. This temperature was essential to provide favorable conditions for the mesophilic anaerobic bacteria. The recycle lines were constructed of polyvinyl chloride tubing while the feed and effluent lines were Tygon and neoprene tubing.

Each AFBR was charged with 2.0 kg of 20x30 U.S. Mesh silica sand serving as the attachment medium. The influent header of each bioreactor was filled with marbles, which distributed the flow evenly across the column cross section. The effluent header captured gas produced during the treatment process, and allowed the liquid effluent to exit the AFBR. All three bioreactors were inoculated with anaerobic bacteria.

Synthetic wastewater

The wastewater fed to the laboratory-scale bioreactors comprised a mixture of three streams: a nutrient stream, a buffer stream, and a stream containing the contaminants and ethanol. These streams were fed into the recycle line using 2 rpm constant speed pump drivers, a model 7018-20 pump head for the feed solution, and a model 7016-20 pump head for the nutrient and buffer solutions (Cole-Palmer Instruments Co., Chicago, IL). The concentrations of the various nutrient and buffer constituents are given in Tables 1 and 2.

Inoculum

Consortia of bacteria were initially seeded from municipal wastewater treatment plant anaerobic supernatant with no enrichments. Previous work (Maloney et al. 2002; VanderLoop et al. 1999; Maloney et al. 1998) always used this approach because the field systems are always open to the environment and thus would be continuously reseeded with various bacteria. The method used here was to provide a broad seed material and then set the conditions of temperature, co-substrate, and nutrient additions to favor anaerobic bacteria.

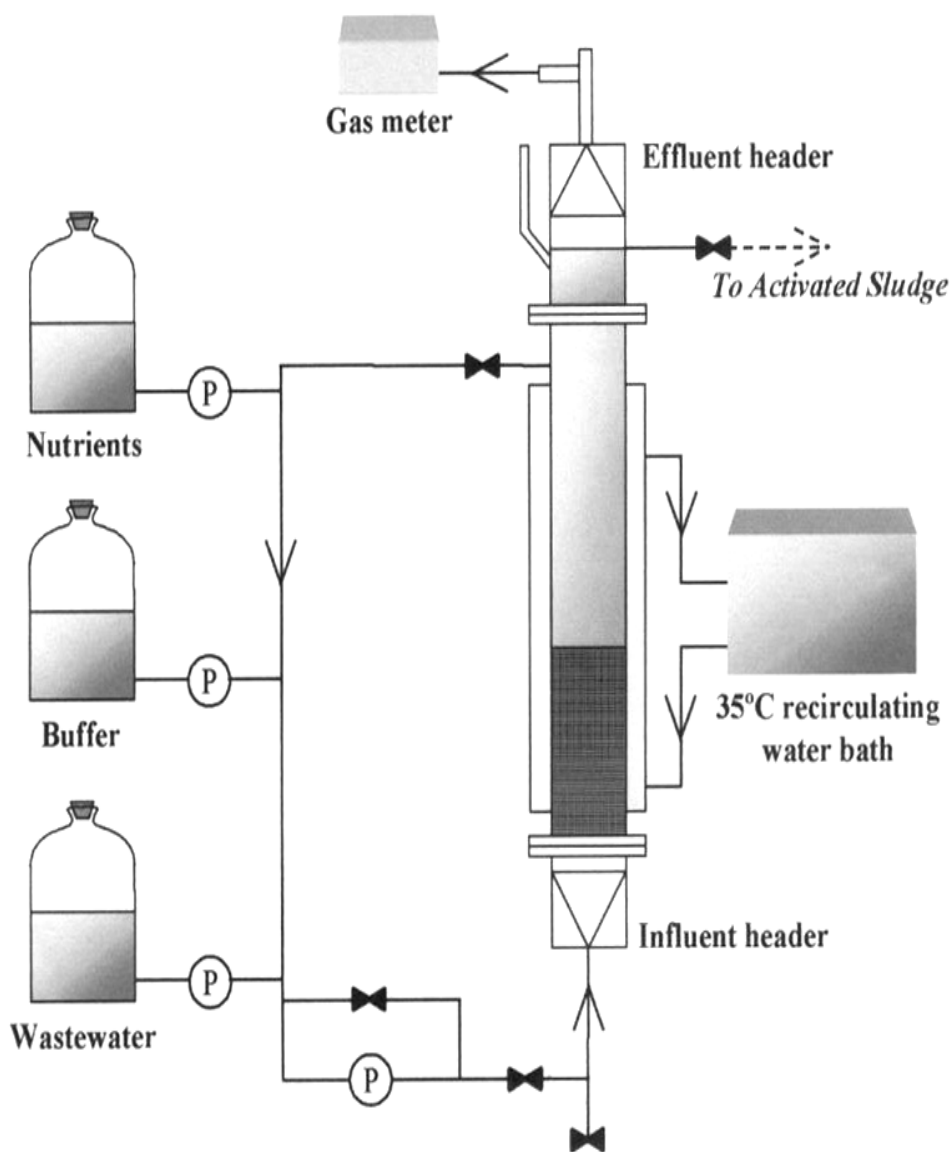


Figure 1. Schematic of anaerobic fluidized bed bioreactor.

Table 1. Concentration of nutrients and vitamins – laboratory-scale study.

Nutrients	Concentration to AFBR (g/L)	Vitamins	Concentration to AFBR (g/L)
CaCl ₂ *2H ₂ O	1.04180	p-Aminobenzoic acid	0.03800
NH ₄ Cl	1.10000	Biotin	0.01500
MgCl ₂ *6H ₂ O	0.50500	Cyanocobalamin (B12)	0.00100
(NH ₄) ₆ Mo ₇ O ₂₄ *4H ₂ O	0.03652	Folic acid	0.01500
Na ₂ B ₄ O ₇ *10H ₂ O	0.00625	Nicotinic Acid	0.03800
NiCl ₂ *6H ₂ O	0.01783	Pantothenic Acid	0.03800
MnCl ₂ *4H ₂ O	0.02790	Pyridoxine hydrochloride	0.07500
CoCl ₂ *6H ₂ O	0.01090	Riboflavin	0.03800
ZnCl ₂	0.01440	Thiamin Hydrochloride	0.03800
CuCl ₂ *2H ₂ O	0.01211	Thioctic acid	0.03800
FeCl ₂ *4H ₂ O	0.12720		

Table 2. Concentration of primary buffer constituents – laboratory-scale study.

Compound	Concentration to AFBR (g/L)
NaOH	As needed to maintain pH 7.2
Na ₂ CO ₃	As needed to maintain pH 7.2
K ₂ HPO ₄	0.336
Na ₂ S*9H ₂ O	0.450

Chemicals

Salts, nutrients, and carbonate were purchased from Fisher Scientific, Pittsburgh, PA, and were of 95% purity or greater. ERDC/CERL provided RDX to the University of Cincinnati. Perchlorate used was in the form of sodium perchlorate, and it was obtained from Fisher Scientific, Pittsburgh, PA (99%+ purity).

Contaminant concentrations

The three bioreactors used in the laboratory-scale study, as described above, were designated P, R, and P&R. The target concentrations of perchlorate and RDX in the final feed to the three bioreactors were 120 and 0 mg/L to reactor-P; 0 and 20 mg/L to reactor-R; and 120 and 20 mg/L to reactor-P&R. The concentration of RDX in bioreactor-R and bioreactor-P&R was decreased to 10 mg/L later in the study (day 274). These feed scenarios allowed for determination of the treatability of the two contaminants both individually and when commingled.

Ethanol concentrations

The concentration of ethanol in the feed to each bioreactor was initially set to 554.77 mg/L (bioreactor-P), 100 mg/L (bioreactor-R), and 565.11 mg/L (bioreactor-P&R). It was later reduced in bioreactor-P and bioreactor-P&R to provide five-fold the concentration of electron donor needed (based on a stoichiometric use of ethanol – see Chapter 4) to achieve reduction of the perchlorate and/or RDX in the influent wastewater (185 mg/L and 195 mg/L, respectively). After steady state was achieved on day 156, the feed concentration of electron donor to the two bioreactors was lowered to 2.5-fold the minimum concentration needed for complete reduction (92.5 mg/L in bioreactor-P and 97.5 mg/L in bioreactor-P&R) (see Appendix A for calculations). The stoichiometric concentration of ethanol to bioreactor-R (see Appendix A for calculations) was too low to maintain anaerobic conditions.

Ethanol concentrations were then varied throughout the study to determine minimum co-substrate requirements. Table 3 lists ethanol feed concentrations used over time.

Perturbation experiments

On days 531, 559, and 601, three identical variations in perchlorate feed were performed. On these days perchlorate was removed from bioreactor-P and bioreactor-P&R for 2 weeks, while bioreactor-R was operated without any change. Perchlorate was introduced back into the feed of bioreactor-P and -P&R on days 544 (1st sampling), 573 (2nd sampling), and 615 (3rd sampling). In addition, bioreactor-R received 120 mg/L of perchlorate on these days as well. Samples were taken every 1 hour for the first 24 hours, and then once or twice each day in a 1-week period.

Oxidation-reduction potential measurements

Oxidation-reduction potential (ORP) was measured before each variation in perchlorate feed using an ORION triode electrode (Thermo Electron Corporation). The ORP standard solution used was quinhydrone, which was added to the pH 4 and 7 buffer solutions in order to create the system of two different potentials (reference points). Potential values of these solutions versus the normal hydrogen electrode (NHE) at 25 °C were 482 mV at pH 4 and 285 mV at pH 7. Nevertheless, quinhydrone solution is relatively unstable, and new standards had to be made before each measurement.

Table 3. Ethanol feed concentrations.

Period	Bioreactor-P EtOH (mg/L)	Bioreactor-R EtOH (mg/L)	Bioreactor-P&R EtOH (mg/L)
Stoichiometric EtOH Concentration	37.00	2 / 1	39 / 38
Acclimation	200	200	200
Day 0 - 51	555	100	565
Day 52 - 155	185	100	195
Day 156 - 231	92.5	50	97.5
Day 232 - 318	92.5	150	97.5
Day 319 - 332	46.2	150	150
Day 333 - 384	92.5	150	150
Day 385 - 414	69.4	200	187
Day 415 - 447	69.4	200	237
Day 448 - 471	69.4	200	300
Day 472-532	69.4	200	400
Day 533-590	69.4	400	400
Day 591-623	69.4	400	550
Day 624-714	69.4	35	550

Analysis

Ion chromatography

Perchlorate was analyzed using the Ion Chromatography DX 500 system (DIONEX Corporation, Sunnyvale, CA). The anion guard column used was DIONEX AG 16 4 mm, and anion separator column – DIONEX AS 16 4 mm. The mobile phase consisted of 100% 50 mM sodium hydroxide, at a flow rate of 1.5 mL/min (EPA Method 314). The detection limit of this instrument was 0.59 µg/L.

High performance liquid chromatography

RDX was analyzed using the high performance liquid chromatography (HPLC) 1100 series with diode array detector, absorbance set at 220 nm (Agilent Technologies, Palo Alto, CA). The column used was ZORBAX SB-C18, 5 µm 4.6 x 250 mm (Agilent Technologies). The mobile phase consisted of water and methanol in a 60:40 ratio at a flow rate of 1.0 mL/min (modifications of EPA Method 8330). The detection limit of this instrument was 0.87 µg/L.

Gas analysis

Effluent gas samples from the AFBRs were analyzed for carbon dioxide, nitrogen, oxygen, and methane with an HP 5890 Series II gas chromatograph (GC) equipped with a thermal conductivity detector (TCD) (Hewlett Packard, Wilmington, DE). The column used was an HP 10-ft molecular sieve BX-45/60 mesh HP 6 ft HAYESEP Q 80/100 (SUPELCO, Bellefonte, PA).

Chemical oxygen demand

Samples were analyzed for chemical oxygen demand (COD) in accordance with the HACH Method 8000, using the HACH DR/2000 Spectrophotometer.

Volatile fatty acids analysis

The volatile fatty acids (VFAs) were analyzed using HP 6890 Series GC (Agilent Technologies, Palo Alto, CA) equipped with flame ionization detector (FID) and 80/120 Carbowax B-DA*/4% Carbowax 20M packed column (SUPELCO, Bellefonte, PA).

Community analysis by 16S rDNA (DGGE)

DNA extraction and PCR conditions

DNA extraction and PCR conditions were as follows:

- Samples from each bioreactor were collected in 50 mL centrifuge tubes and centrifuged at 10,000X g for 10 min,
- The supernatant was decanted and the samples were stored in -80 °C for later analysis by DGGE,
- Genomic DNA was extracted from each sample using the Ultraclean soil DNA extraction kit (Mo Bio Laboratories, Solana Beach, CA) according to the manufacturer's instructions.
- The isolated genomic DNA was used as template material for the polymerase chain reaction (PCR).
- The variable V3 region of the bacterial 16S ribosomal DNA (rDNA) was amplified by PCR.
- PCR was performed in 50 µL reaction volume using a reaction mixture of 1X PCR buffer, 200 µM each deoxynucleoside triphosphate, 2.5 mM magnesium chloride (MgCl₂), 0.025 units of Taq DNA polymerase/µL (Qiagen, Valencia, CA), and 0.5 µM of each primer.

The primers used were 341F (5'-CCTACGGGAGGCAGCAG-3') and 518R (5'-ATTACCGCGGCTGCTGG-3') with a GC clamp attached to the 5' end of the forward primer (manufactured by the University of Cincinnati DNA Core lab) (Muyzer et al. 1993). Amplification of DNA was performed in a GeneAmp PCR system 2400 (Perkin Elmer) by using the following program: an initial denaturing step at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 1 min and 30 s, and final extension at 72 °C for 10 min. PCR tubes were placed in the thermocycler when the block temperature reached 94 °C.

DGGE analysis

DGGE was performed on a D-code system (BioRad, Hercules, CA). Equal amounts of DNA (approximately 500 ng) were loaded onto 8% polyacrylamide gel (37.5:1, acrylamide:bisacrylamide) with a 30 to 60% linear gradient of denaturant (100% denaturant contains 7 M urea and 40% [vol.vol] formamide). Gels were run for 20 h at 35 V in 0.5XTAE buffer (20 mM tris-acetate and 0.5 mM ethylene diamine tetraacetic acid (EDTA) at pH 8.0) maintained at 60 °C. Following electrophoresis, the gel was stained for 20 min with SYBR Green I (10 µL in 100 mL of 1XTAE) (Molecular Probes, Eugene, OR) and visualized by ultraviolet illumination. The gel images were documented using the Kodak 1D Image Analysis Software (Eastman Kodak, Rochester, NY).

Sequencing analysis

Dominant DGGE bands were excised from the gel and purified using a QIquick Gel Extraction Kit (QIAGEN, Valencia, CA) according to instructions of the manufacturers. Purified DGGE bands were sent for sequencing to the Cincinnati Children's Hospital Sequencing Laboratory.

Demonstration-scale GAC-FBR

A near full-scale system, designated bioreactor-D in this report, was installed at MCAAP under the Environmental Security Technology Certification Program (ESTCP) for the treatment of pinkwater. A system description and the results obtained have been described elsewhere in detail (Maloney et al. 2002; ESTCP 2003). A schematic diagram of the system is shown in Figure 2. The system primarily consists of a 4.5-ft diameter column that is 22 ft tall, and a 3-ft diameter separator that is 5 ft tall. The separator collects biomass and GAC that wash out of the fluidized bed and returns them to the 22-ft column. Two modifications were made to the system from the ESTCP study.

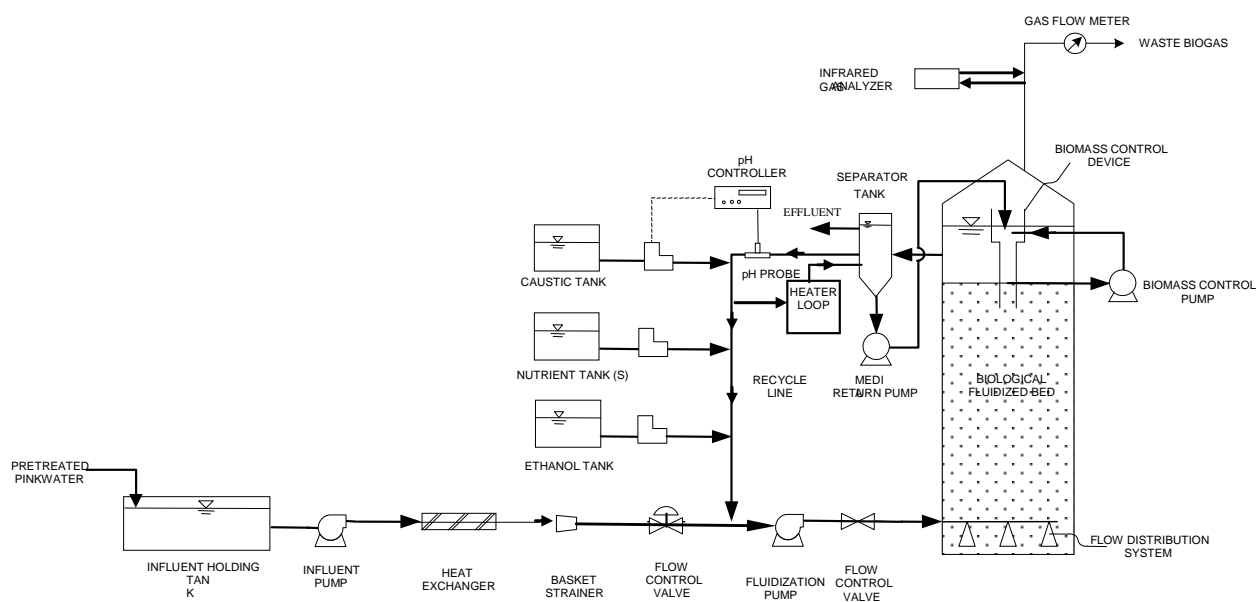


Figure 2. Schematic diagram of demonstration-scale GAC-FBR at MCAAP.

The first modification was made to correct hydraulic problems associated with the separator. It was originally designed to be near ground level and discharge to an effluent transfer tank. This design broke the hydraulic gradeline and depended a great deal on pumps. The separator was moved to an elevated position, which allowed it to discharge by gravity (eliminating the effluent collection tank and effluent discharge pump).

The second change involved micronutrients used to support the anaerobic bacteria. A review of the literature shows that the mix of micronutrients, and sometimes vitamins, varies from researcher to researcher. When working at an industrial plant, it also becomes expensive. This expense is not due to the underlying cost of the chemicals, which is quite low, but rather to the personnel cost for measuring out and packaging the chemicals for use by the wastewater operators. Instead of a custom mix, a readily available, mass-produced product intended for cattle feed supplements (Opticor Range Minerals produced by Archer Daniels Midland, Decatur, IL) was used.

The demonstration plant has been operating for more than 2 years on a continuous basis, except for occasional shutdowns. The system is run by a programmable logic controller, with a touch screen for changes in operating parameters. Shutdowns occur when electricity is interrupted, when

heat is lost (heat is supplied by steam, and the steam plant is occasionally shut down), or when some aspect of the plant operation goes beyond a specified range. Control of pH is provided by caustic addition based on feedback control from in-line probes.

The touch screen is used to change flow rates and change concentrations of target contaminants. A comparison of the hydraulic and contaminant loading characteristics of the bench and demonstration-scale system is shown in Table 4. As the wastewater characteristics change, the data is input via the touch screen. TNT and RDX have occasionally broken through to the effluent, at which time the reactor is placed in recycle until the biomass recovers.

Comparison of the numbers in Table 4 shows that the hydraulic retention times were similar between the bench- and demonstration-scale systems. Although the demonstration-scale system (bioreactor-D) had been operated with a flowrate as high as 6 gpm, it was operated at 1-2 gpm during the perchlorate campaign tests, because there was insufficient storage to hold the wastewater at higher flowrates. As previously described, all of the wastewater had to be held, and recycled, until the perchlorate concentration was below detection limits, so that no perchlorate would be discharged to the industrial wastewater treatment plant. Thus the hydraulic retention time was 44 hours (at 1 gpm), which is slightly longer than the bench tests, to 22 hours (at 2 gpm), which was shorter than the laboratory-scale tests.

RDX loading in the demonstration-scale (bioreactor-D) tests was consistently lower than the laboratory-scale (bioreactors-R and -P&R) tests, but TNT loading was occasionally high in the demonstration-scale tests, while it was absent in the laboratory-scale tests. Perchlorate loadings achieved in the laboratory-scale tests were much higher (by a factor of 5) than ever tested in the demonstration-scale tests.

Tests of perchlorate degradation in bioreactor-D were performed by introducing a concentrated feed stream of perchlorate dissolved in plant water into the feed line of the FBR. No specific mixing was used, but the 1.5-in. diameter feed line was approximately 12 ft long from the injection point to the connection to the recirculation line of the FBR. Concentration targets were set, and the concentration used in the perchlorate feed was calculated to achieve those targets, but actual concentrations as determined by the influent water analysis were used in this study.

Table 4. Comparison of bench- and demonstration-scale reactors.

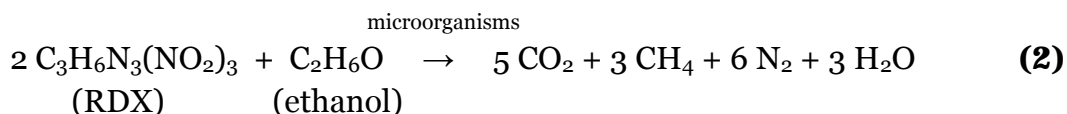
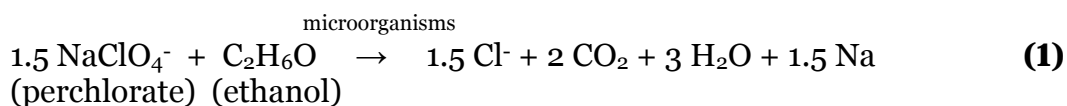
Parameter	Bench Test	Demonstration Reactor
Flow rate	6 L/day	1-6 gpm (5450-32,700 L/day)
Reactor Volume	9.1L	2,600 gal (9,900 L)
Hydraulic Retention Time	36 hours	44 to 7 hours (@1-6 gpm)
Support Medium	sand	granular activated carbon
Organic Loading Rate		
Contaminant Concentration		
- Perchlorate mg/L	120	5-30
- RDX mg/L	10-20	1-5
- TNT mg/L	0	10-150
Contaminant Loading Rate		
- Perchlorate g/m ³ -day	79	5.5-16.5
- RDX g/m ³ -day	6.6-13.2	0.6-5.5
- TNT g/m ³ -day	-	5.5-165

Tests at the demonstration-scale were confined to short-term “campaigns,” during which all of the effluent was collected in two 5000-gal effluent tanks. Effluent collection allowed recirculation of the water after the campaign was finished, so that the concentration of perchlorate would be reduced to below detection limits before the wastewater was discharged to the industrial plant on site at MCAAP.

4 Results and Discussion

Ethanol requirements

The ethanol needed to reduce perchlorate and RDX was calculated assuming complete mineralization of these two compounds. These calculations represent the minimum requirements possible. Ethanol would also be required to sustain bacterial growth and reduce other oxidants present such as oxygen and nitrate. The assumed mineralization reactions for the two compounds are as follow:



The amount of ethanol needed for the stoichiometric reduction of the two contaminants is presented in Appendix A. During the acclimation period (from the start up of the reactors until the addition of the contaminants), the concentration of ethanol fed to the reactors was 200 mg/L. During this time, feed to the bioreactors was introduced using two reservoirs, one containing nutrient (flow rate of 0.5 L/d), and the other containing ethanol and buffer in super Q water (flow rate 5.5 L/d).

The combined flow rates from the two reservoirs were increased gradually from 1/4 of the target 6 L/d, to 1/2 and eventually to the full flow of 6 L/d. On day 0, perchlorate and RDX were added to the feed solution, in concentrations of 120 mg/L and 20 mg/L, respectively (concentration of RDX in bioreactor-R and bioreactor-P&R was decreased to 10 mg/L on day 274). The ethanol concentration was increased to 554.77 mg/L in bioreactor-P and 565.11 mg/L in bioreactor-P&R; it was decreased to 10.36 mg/L in bioreactor-R. These concentrations were based on erroneous stoichiometric calculations for the electron donor, and were corrected on day 52. Application of these ethanol concentrations to the bioreactor-R resulted in very poor reactor performance; consequently, the concentration of ethanol was increased to 100 mg/L.

During the acclimation period, some of the nutrients were observed to be precipitating in the feed reservoir. To rectify this problem, a three feed reservoir scenario was adopted. Sodium sulfide, potassium hydro phosphate, and sodium carbonate were taken out of the stock solution and placed in a separate reservoir. Sodium hydroxide was added in order to control the pH at 7.2. Flow rate of this solution and the flow rate of the nutrient stream were set at 0.5 L/d each, while the flow rate of the stock solution, containing the ethanol and the contaminants, was reduced to 5 L/d, resulting in a total flow rate of 6 L/d. On day 52, the feed ethanol concentrations to bioreactor-P and bioreactor-P&R were increased to five-fold the stoichiometric electron donor demand (the new concentrations of 185 mg/L in bioreactor-P and 195 mg/L in bioreactor-P&R).

Since bioreactor-R previously showed poor performance under low concentrations of electron donor, the feed ethanol concentration to this reactor was maintained at 100 mg/L. After reaching steady state, the feed ethanol concentration to bioreactor-P and bioreactor-P&R were reduced to 92.5 mg/L and 97.5 mg/L, respectively. The feed ethanol concentration to bioreactor-R was decreased to 50 mg/L (days 156–231), and, after steady state was later attained, the concentration of electron donor was increased to 150 mg/L (day 232). In order to test the efficiency of RDX biodegradation under higher concentrations of electron donor, the concentration of ethanol in the final feed of bioreactor-P&R was increased to 150 mg/L (day 319).

Since bioreactor-P exhibited good performance in biodegrading perchlorate under lower ethanol concentrations, the concentration of the electron donor in the feed to this reactor was reduced by half on day 319 (46.25 mg/L). This reduction of electron donor, close to the stoichiometric amount, resulted in a poor performance of bioreactor-P, and hence the concentration was increased again to 92.5 mg/L (day 333). After steady state was attained, concentration of ethanol in bioreactor-P was decreased to 69.375 mg/L (day 385). Reactor operation under this electron donor concentration went without any complications until the end of the study.

To test if a higher concentration of ethanol would further reduce the amount of RDX in the effluent of bioreactor-R, the concentration of electron donor was increased to 200 mg/L (day 385). On the same day, the concentration of electron donor in bioreactor-P&R was raised to 187 mg/L. To test the impact of higher concentrations of electron donor on the reduction of RDX in bioreactor-P&R, the concentration of ethanol was raised

several more times; to 237 mg/L on day 415, to 300 mg/L on day 448, and to 400 mg/L on day 472.

To acclimate the microbial culture in bioreactor-R to the introduction of perchlorate during the three perturbation events, the concentration of ethanol in this bioreactor was raised to 400 mg/L (day 533). Right before the third perturbation, the concentration of ethanol in bioreactor-P&R was increased to 550 mg/L (day 591). The final change in ethanol concentration occurred on day 624 when the influent concentration of electron donor to bioreactor-R was decreased to 35 mg/L.

Chemical oxygen demand analysis

Figures 3, 4, and 5, present influent and effluent COD concentrations for bioreactor-P, bioreactor-R, and bioreactor-P&R, respectively. A detailed tabulation of the effluent quality data for each column can be found in Appendix B. Effluent COD can be used as a measure of the “health” of the biomass. Once biomass is established, effluent COD should remain at a relatively low and constant value. If something (such as a toxic shock) occurs, effluent COD may suddenly rise, indicating the system is losing effectiveness.

Bioreactor-P

Influent COD varied with the ethanol concentration. A slight drop in influent COD concentration was observed during the period between days 319 and 332 corresponding to a decrease in the influent ethanol concentration to 46.25 mg/L. This influent COD concentration increased when the influent concentration of ethanol was increased again to 92.5 mg/L on day 333. After reducing the concentration of electron donor to 69.5 mg/L on day 385, the influent COD concentration dropped to approximately 150 mg/L. Influent COD concentrations for the period between days 0 and 51 were not measured.

The effluent COD concentration from bioreactor-P averaged 36 mg/L during the period between days 0 and 51. This effluent COD concentration decreased to an average of 14 mg/L for the period between days 52 and 155. The effluent COD concentration decreased further during the period between days 156 and 318, when the feed ethanol concentration was lowered to 92.5 mg/L. After day 318, the effluent COD concentration remained at an average of 12 mg/L regardless of any further changes in the feed

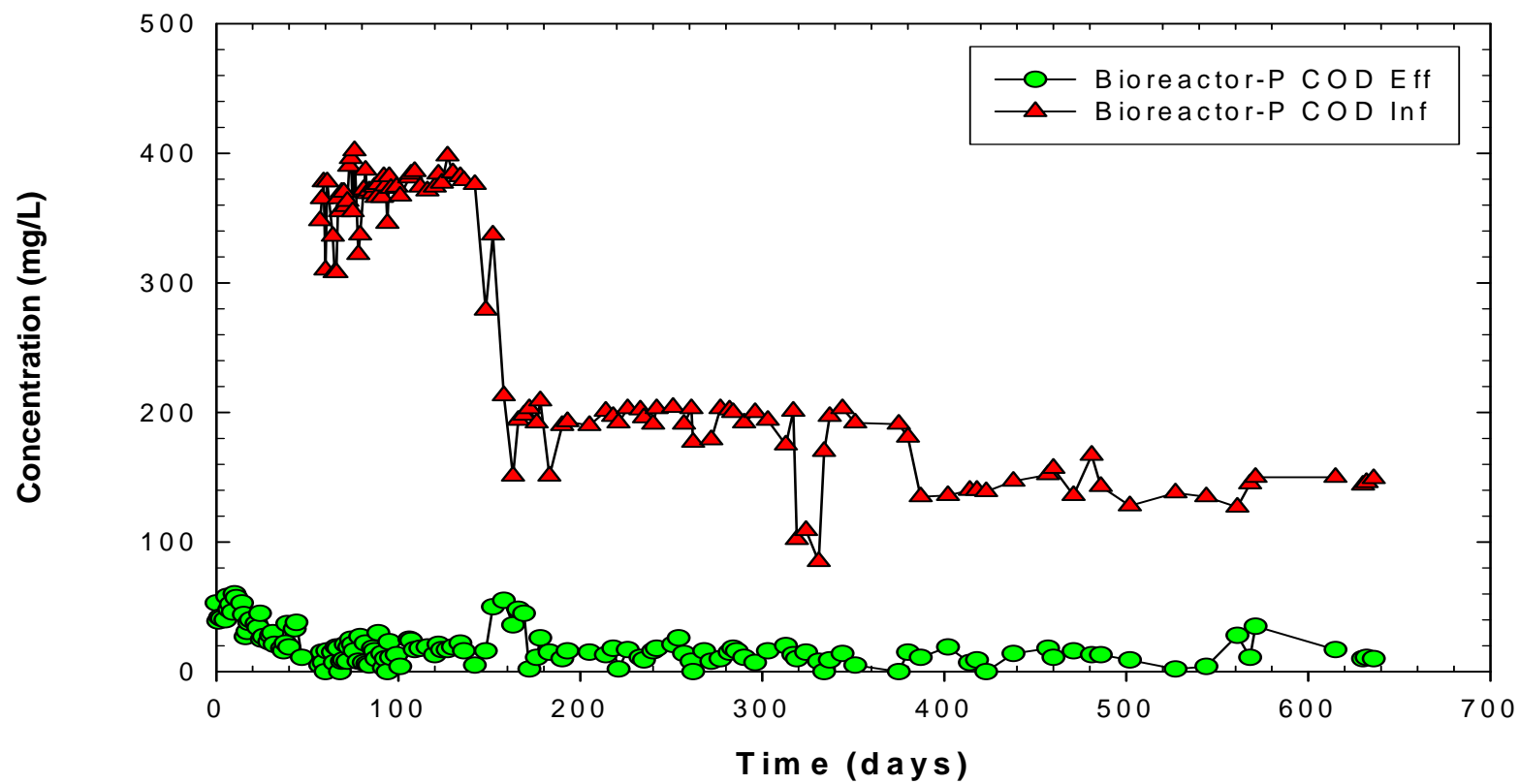


Figure 3. Bioreactor-P COD concentrations.

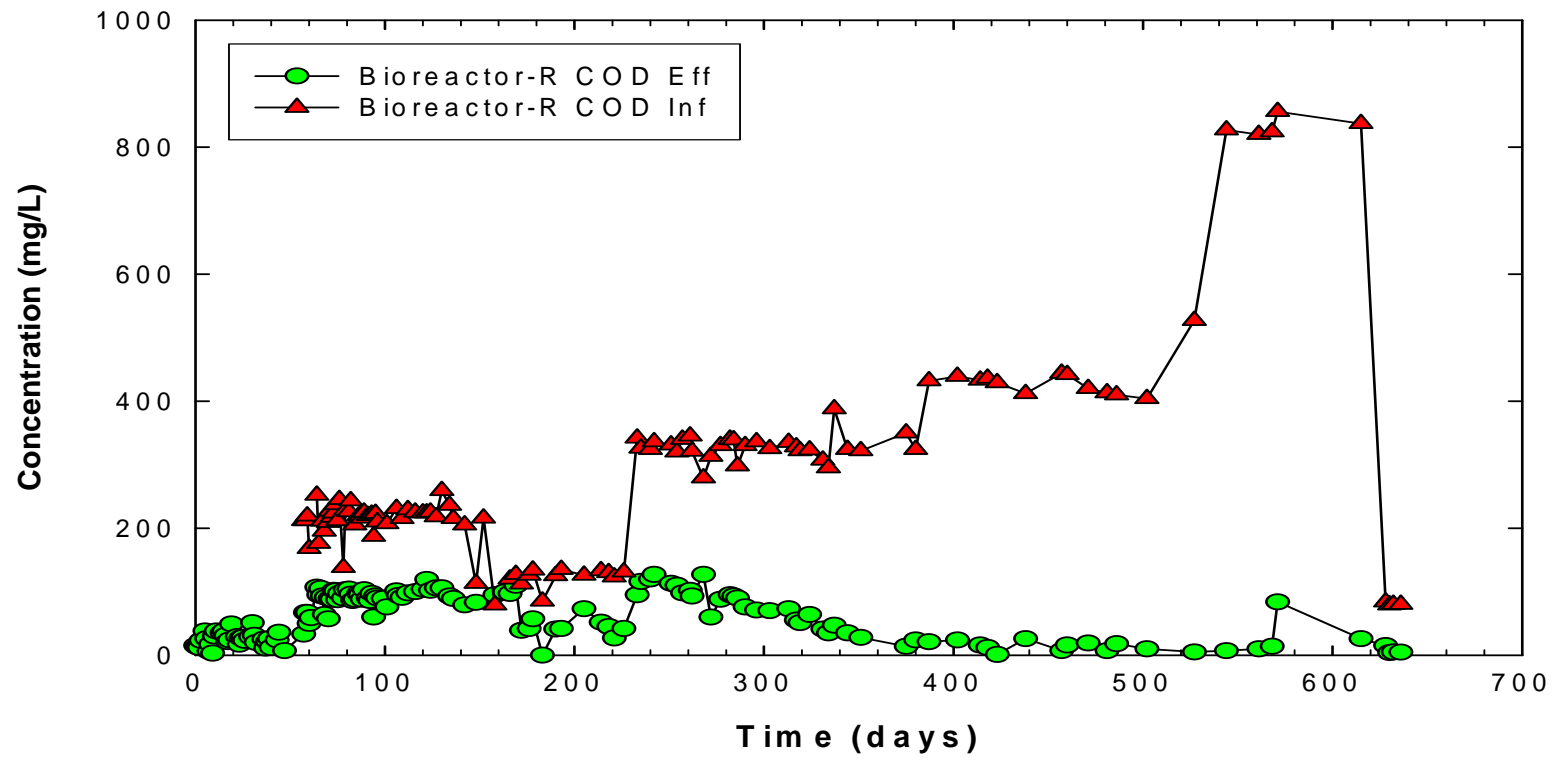


Figure 4. Bioreactor-R COD concentrations.

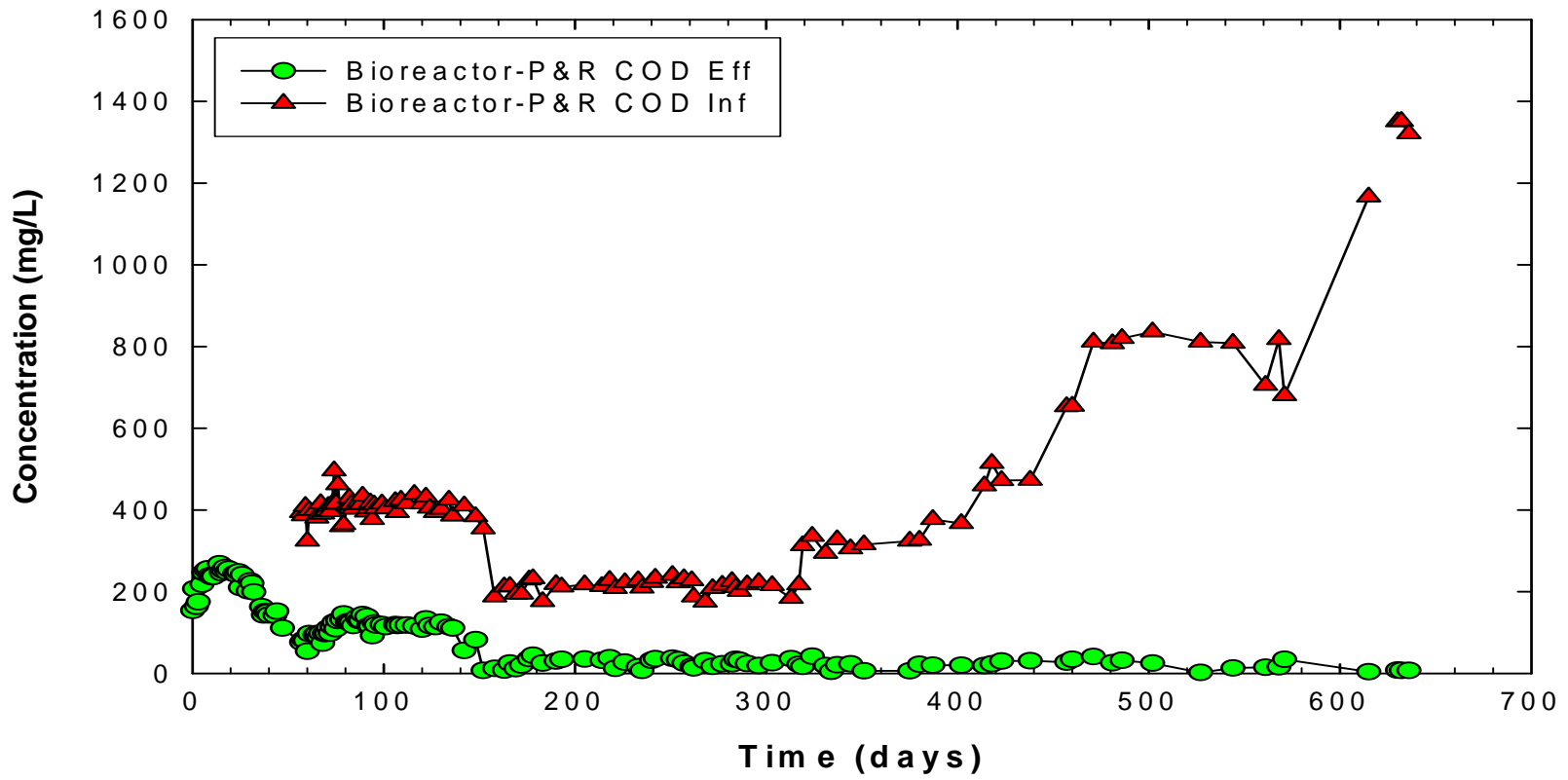


Figure 5. Bioreactor-P&R COD concentrations.

concentration of ethanol. This average indicates that the biomass remained healthy throughout the study.

Bioreactor-R

For bioreactor-R (Figure 4), COD concentrations were significantly lower during the period prior to day 52, than during the period when RDX was initially added between days 52 and 155 (average COD concentrations were 25 and 88 mg/L, respectively). These averages indicate that RDX exerted a toxic shock to Bioreactor R when first introduced. This average was in spite of the fact that the influent ethanol concentration to bioreactor-R was 100 mg/L throughout the two periods. After the influent ethanol concentration to bioreactor-R was reduced to 50 mg/L, a corresponding decrease in the effluent COD concentrations was observed (averaging 42 mg/L).

The effluent COD increased to an average of 62 mg/L corresponding to an increase in the feed ethanol concentration 150 mg/L on day 232. Further increases in the ethanol feed concentrations to 200 mg/L (days 385–532) and 400 mg/L (days 533–623) resulted in decreases in the effluent COD concentration to approximately 14 mg/L and 28 mg/L, respectively, suggesting that healthy anaerobic activity was sustained under higher loadings of ethanol, which may be a result of the biomass acclimating to RDX. As discussed later in the section on RDX removal, RDX effluent concentrations did not decline to acceptable levels until day 274. By that time, the biomass acclimated and was able to produce effluent low in both COD and RDX concentrations. When the influent concentration of ethanol was decreased to 35 mg/L, the effluent COD concentration averaged 9 mg/L. Once again, influent COD concentrations were not measured for the first 51 days of operation.

Bioreactor-P&R

Higher effluent COD concentrations were observed for bioreactor-P&R (Figure 5) during the period extending from days 0 to 51. After this initial period, effluent COD concentrations started to generally decline while corresponding to changes in the influent ethanol concentration. When the ethanol concentration was reduced to five-fold the corrected stoichiometric demand (days 52–155), the effluent concentration of COD averaged 108 mg/L. Another drop in the effluent COD concentrations averaging 27 mg/L was observed during the period between day 156 and day 318,

when the influent ethanol concentration was reduced to 97.5 mg/L. The sharp drop was reflected in the influent concentrations as well.

When the influent concentration of ethanol was increased to 150 mg/L between days 319 and 384, the effluent COD concentration decreased further to an average of 15 mg/L, suggesting that improved methanogenic activity was taking hold within bioreactor-P&R, which also suggests acclimation to the RDX. When the ethanol concentration was raised to 187 mg/L, effluent COD concentration slightly increased to approximately 20 mg/L, which indicates that a healthy biomass acclimated to RDX and perchlorate had been obtained. Despite the further increase in the concentration of electron donor, effluent COD concentration remained at 20 mg/L, which indicates that a healthy biomass acclimated to RDX and perchlorate had been obtained. Only when the concentration of ethanol was increased to 550 mg/L (day 591) did effluent COD concentration drop to approximately 10 mg/L.

Summary

In general, it can be concluded that the three bioreactors required a minimum influent concentration of the electron donor for proper methanogenic activity to prevail. This minimum concentration can be a function of the presence of the energetic compound, any dissolved oxygen in the feed, and the amount of oxygen that can diffuse into the reactor through Tygon tubing of the Plexiglas used in reactor construction.

Perchlorate removal

Figures 6 and 7 show the performance of bioreactor-P, which receives perchlorate (ClO_4^-), and bioreactor-P&R, which receives both ClO_4^- and RDX, relative to perchlorate removal. As seen from Figure 6, during the period between days 52 and 150 of perchlorate feeding, the concentration of this contaminant in the effluent of bioreactor-P was variable, even though the operating conditions were maintained constant (steady influent concentrations of 120 mg/L). During this period, the concentration of ethanol was 5 times the stoichiometric concentration needed to reduce the feed

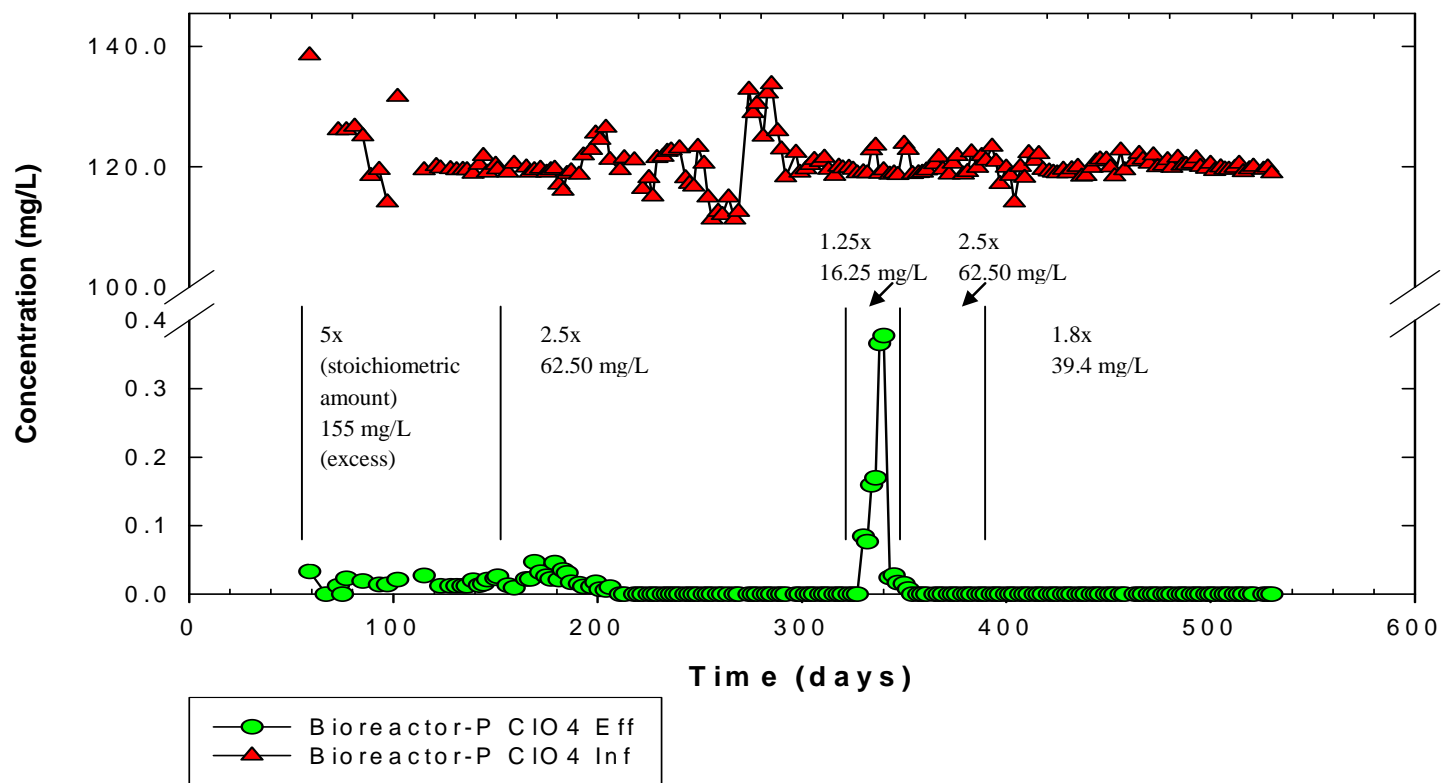


Figure 6. Bioreactor-P perchlorate reduction prior to perturbations.

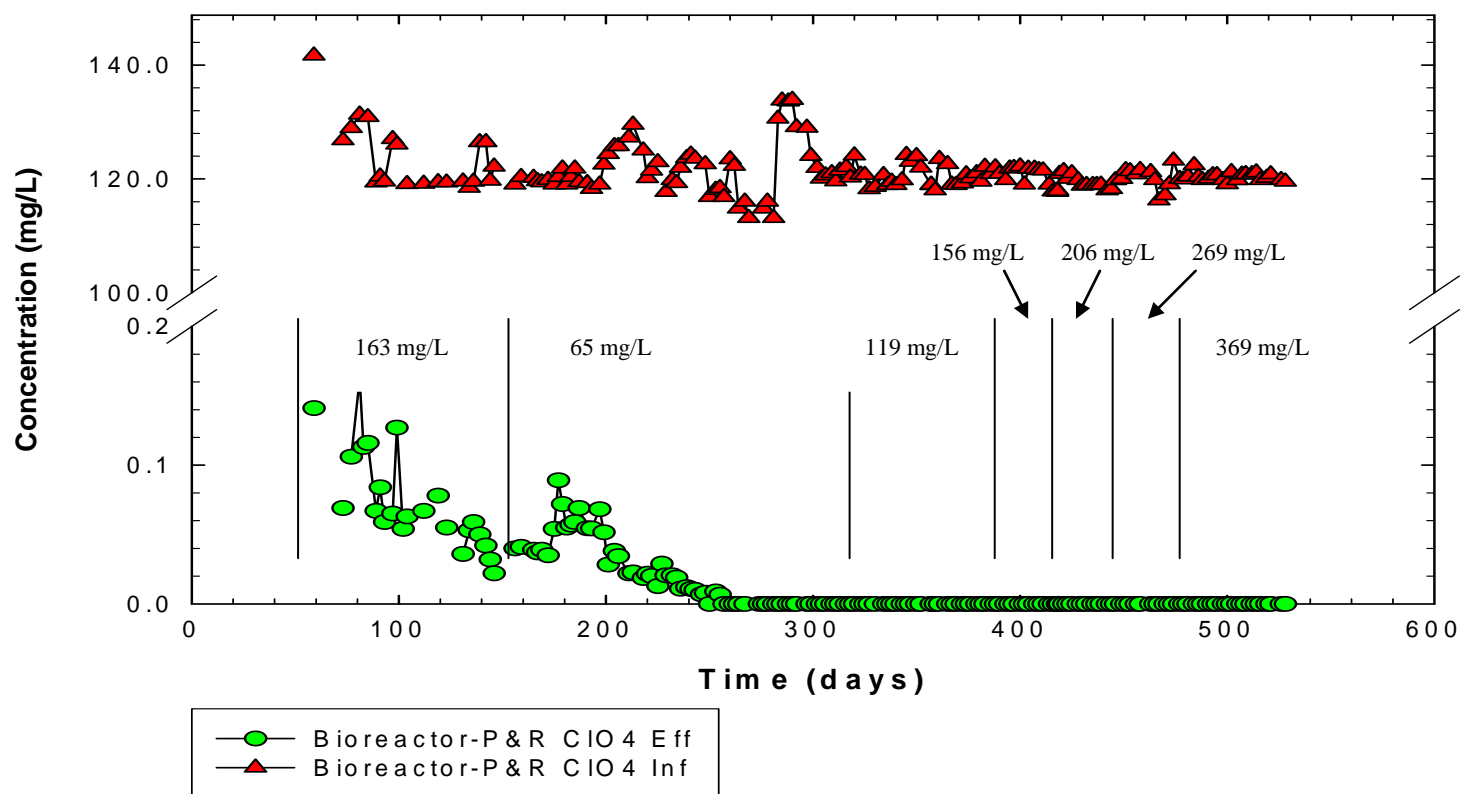


Figure 7. Bioreactor-P&R perchlorate reduction prior to perturbations.

perchlorate. After day 156, the effluent concentration of perchlorate stabilized at approximately 20 µg/L.

At this point (day 156), the feed concentration of ethanol was reduced to 2.5 times the stoichiometric amount. This decrease in feed ethanol resulted in a temporary slight increase in the effluent concentrations of perchlorate, which subsequently decreased to below the detection limit. Based on this behavior, concentration of ethanol was further reduced to 46 mg/L (days 319–332), which is close to (1.25 times) its stoichiometric amount. This sharp decrease in the concentration of electron donor caused the concentration of perchlorate in the effluent to increase from below the detection limit to over 400 µg/L.

Due to this deterioration in reactor performance, the concentration of ethanol in the feed to bioreactor-P was increased again to 2.5 times its stoichiometric amount. Recovery of the bioreactor was very rapid. The concentration of perchlorate in the final effluent decreased to below the detection limit within 2 weeks of operation under the restored electron donor condition. Even when the concentration of ethanol was reduced to 1.8 times its stoichiometric demand (day 385) the effluent perchlorate concentration remained below detection limit.

A somewhat different scenario was observed in bioreactor-P&R (Figure 7). During the initial period of reactor operation, the concentration of ethanol in the feed was 5 times its stoichiometric amount, which resulted in a gradual decrease in effluent perchlorate concentrations from 150 to approximately 70 µg/L. After steady-state operation was attained (day 156), the concentration of ethanol was reduced to 2.5 times the stoichiometric amount. This reduction resulted in a small increase in effluent perchlorate concentration, which soon after decreased to below the detection limit.

The responses of bioreactor-P and bioreactor-P&R to various concentrations of ethanol suggest that the efficiency of perchlorate reduction is not very sensitive to the influent concentration of electron donor, provided that concentration exceeds the stoichiometric demand of the energetic compounds present in the feed as well as the demand for electron donor needed to reduce any oxygen entering the anaerobic bioreactors. Figures 6 and 7 show excess amounts of electron donor (ethanol) in mg/L.

Perturbation experiments

The concentrations of perchlorate in the final effluent from bioreactor-P and bioreactor-P&R, remained below detectable levels until day 544 when the first of the three perturbations in feed was performed (data were not collected for any of the feed perturbations during the 2 weeks of no perchlorate in the feed to these two bioreactors). All three feed perturbations for bioreactor-P are represented in Figure 8. As can be seen from the first feed perturbation event, the effluent concentration of perchlorate increased from below detection limits to approximately 800 µg/L within 1 hour after perchlorate had been reintroduced into the system. The effluent concentration of perchlorate continued to increase rapidly, reaching a level of 3300 µg/L 5 hours after resumption of perchlorate feed to the bioreactor. Over the next 3 hours, the concentration of perchlorate remained in the neighborhood of 3000 µg/L, after which a gradual drop was observed. The effluent concentration of perchlorate decreased to below detectable levels 58 hours after the initial reintroduction of perchlorate in the feed.

The second feed perturbation event took place on day 573. Similarly to what was observed during the first perturbation, effluent concentrations of perchlorate were as high as 1200 µg/L 1 hour after perchlorate was reintroduced in the feed. The highest effluent concentration of 2500 µg/L was reached 5 hours after the start of the experiment. After this point, a gradual decrease in effluent perchlorate concentration was observed, reaching levels below the detection limit 48 hours from the initial start of the experiment.

A similar response was observed during the third sampling event (day 615). In the first hour after perchlorate reintroduction, the concentration of perchlorate in the final effluent increased from below the detection limit to approximately 1200 µg/L. The perchlorate concentration continued to rise, and 6 hours after the start of the experiment the concentration in the final effluent was approximately 1700 µg/L, which was significantly lower compared with the previous two experiments. After this point, effluent perchlorate concentration started to drop gradually, reaching concentrations below detection limits 48 hours after the start of the experiment.

A distinctly different response was observed in the bioreactor that was treating the two contaminants perchlorate and RDX (bioreactor-P&R). As can be seen in Figure 9, when perchlorate was reintroduced into the feed, effluent concentration of perchlorate increased to 1000 µg/L within

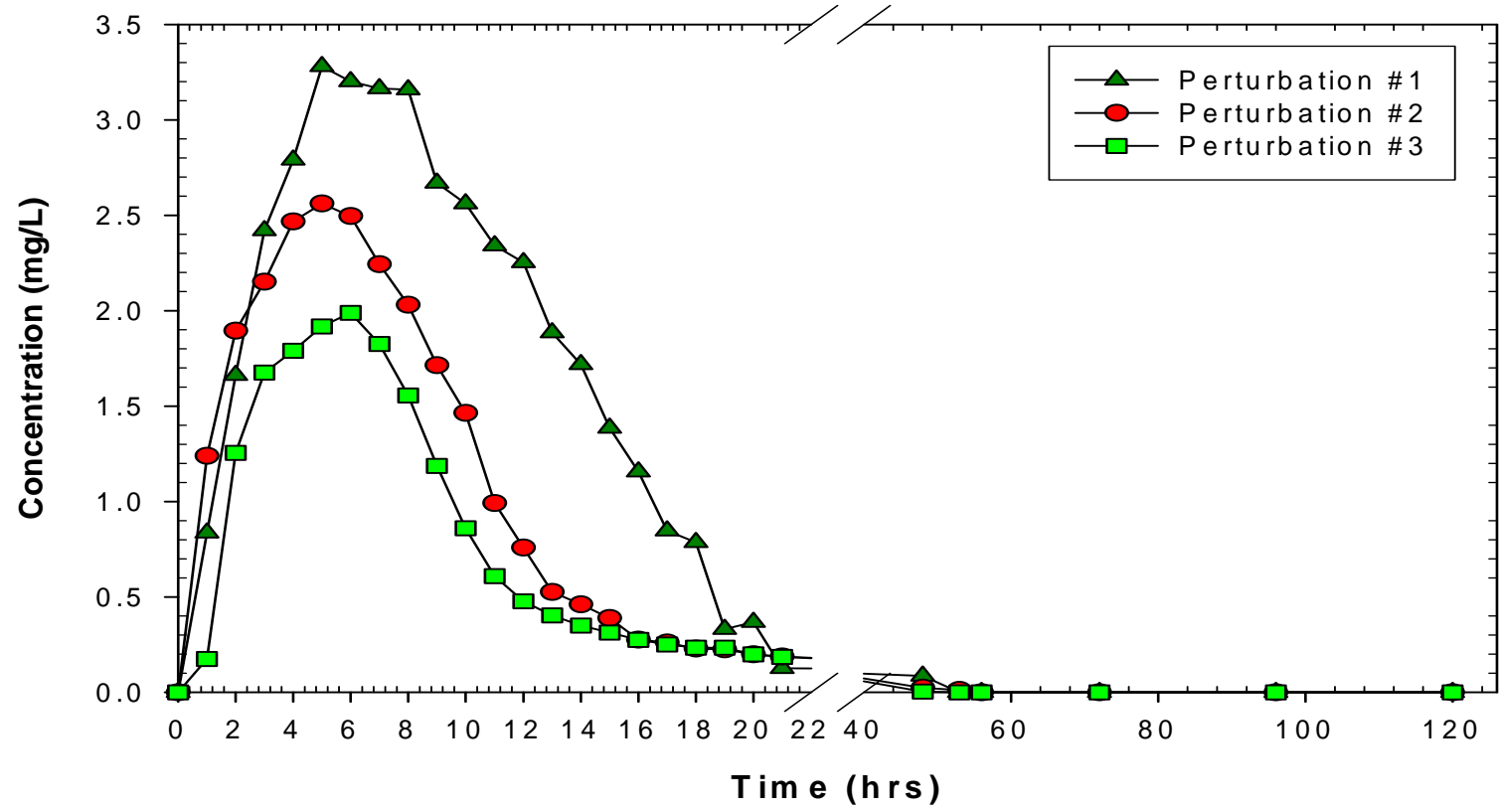


Figure 8. Bioreactor-P perchlorate reduction during perturbations.

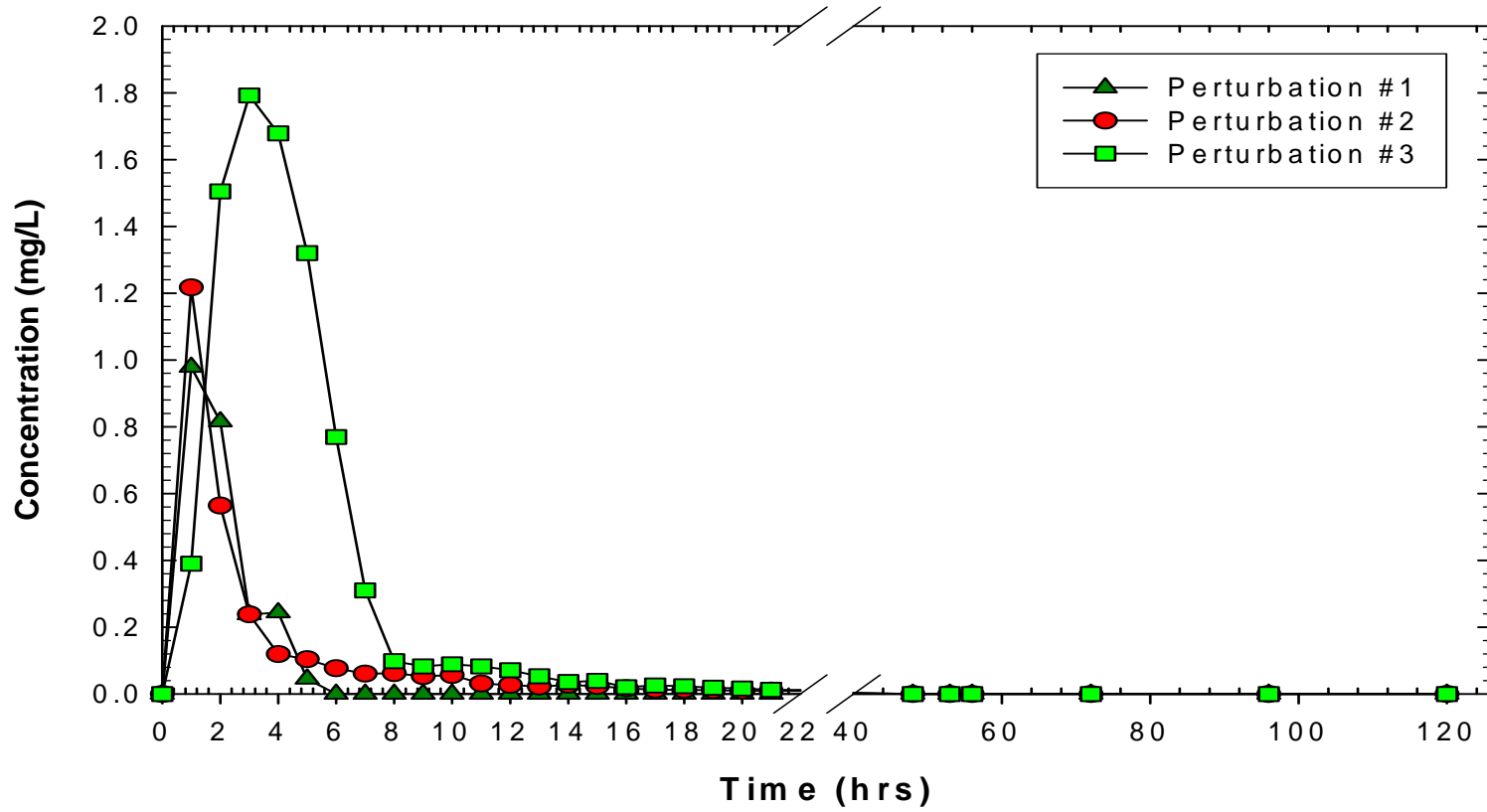


Figure 9. Bioreactor-P&R perchlorate reduction during perturbations.

1 hour. After this point, concentration of perchlorate in the final effluent decreased rapidly to below the detection limit within 6 hours after the initial start of the experiment.

The second perturbed feeding experiment on bioreactor-P&R followed a somewhat similar pattern. Concentrations of perchlorate increased from below the detection limit to approximately 1200 µg/L during the first hour of sampling, after which concentrations of perchlorate decreased rapidly to approximately 100 µg/L after 4 hours. After this point, the drop in concentration of perchlorate was more gradual reaching concentrations below the detection limit 21 hours after the initial start of the experiment.

The highest rise in effluent perchlorate concentrations was observed during the third sampling event. Within the first 3 hours of the experiment, the concentrations of perchlorate increased from below the detection limit to approximately 1800 µg/L. After reaching this peak, the perchlorate concentrations decreased rapidly to a concentration of approximately 100 µg/L (8 hours), after which this drop became more gradual until reaching concentrations below detection limit 21 hours after the initial start of the experiment.

Bioreactor-R exhibited a distinctly different behavior from the other two bioreactors relative to its response to the sudden introduction of perchlorate to its feed. This bioreactor was never exposed to perchlorate over the entire duration of the steady-state operating period of the study. Consequently, when perchlorate was introduced to the feed, its concentration in the final effluent started to increase significantly (Figure 10). To avoid potential reactor failure, perchlorate was removed from the feed 8 hours after its initial introduction to the system. However, its concentration in the effluent continued to increase for an additional 3 hours, reaching concentrations over 29,000 µg/L. Twelve hours after the startup of the experiment, a gradual decrease in effluent concentrations was observed. The concentration of perchlorate in the effluent decreased to below its detection level 120 hours into this experiment.

A similar pattern of behavior was observed for the second and third feed perturbation experiment for bioreactor-R (Figures 11 and 12). After the initial introduction of perchlorate into the system, a dramatic increase in its effluent concentration was observed (42,000 µg/L for the second perturbation and 38,000 µg/L for the third perturbation). The contaminant

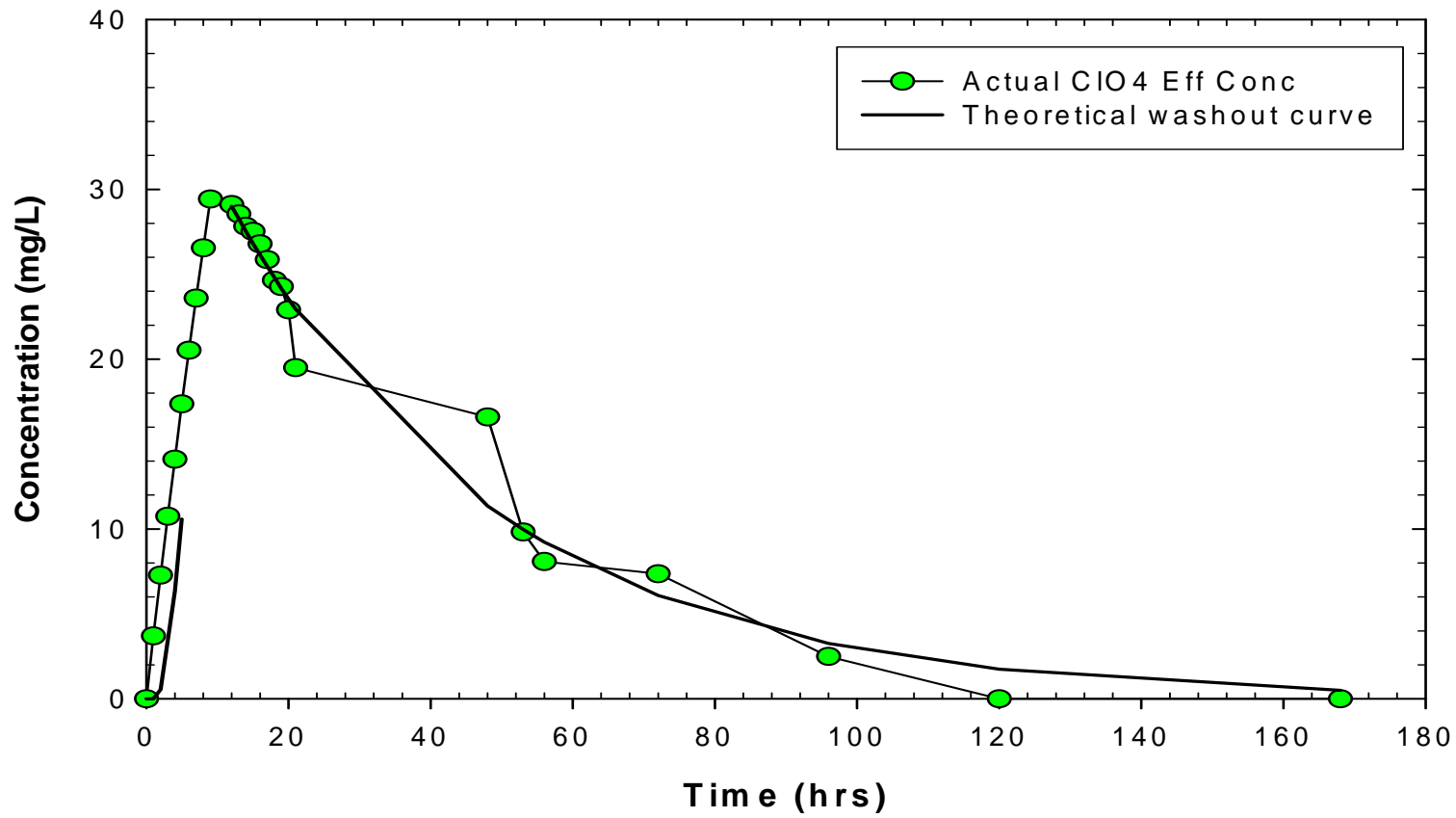


Figure 10. Bioreactor-R comparison of $[\text{ClO}_4^-]$ to the washout curve (first perturbation).

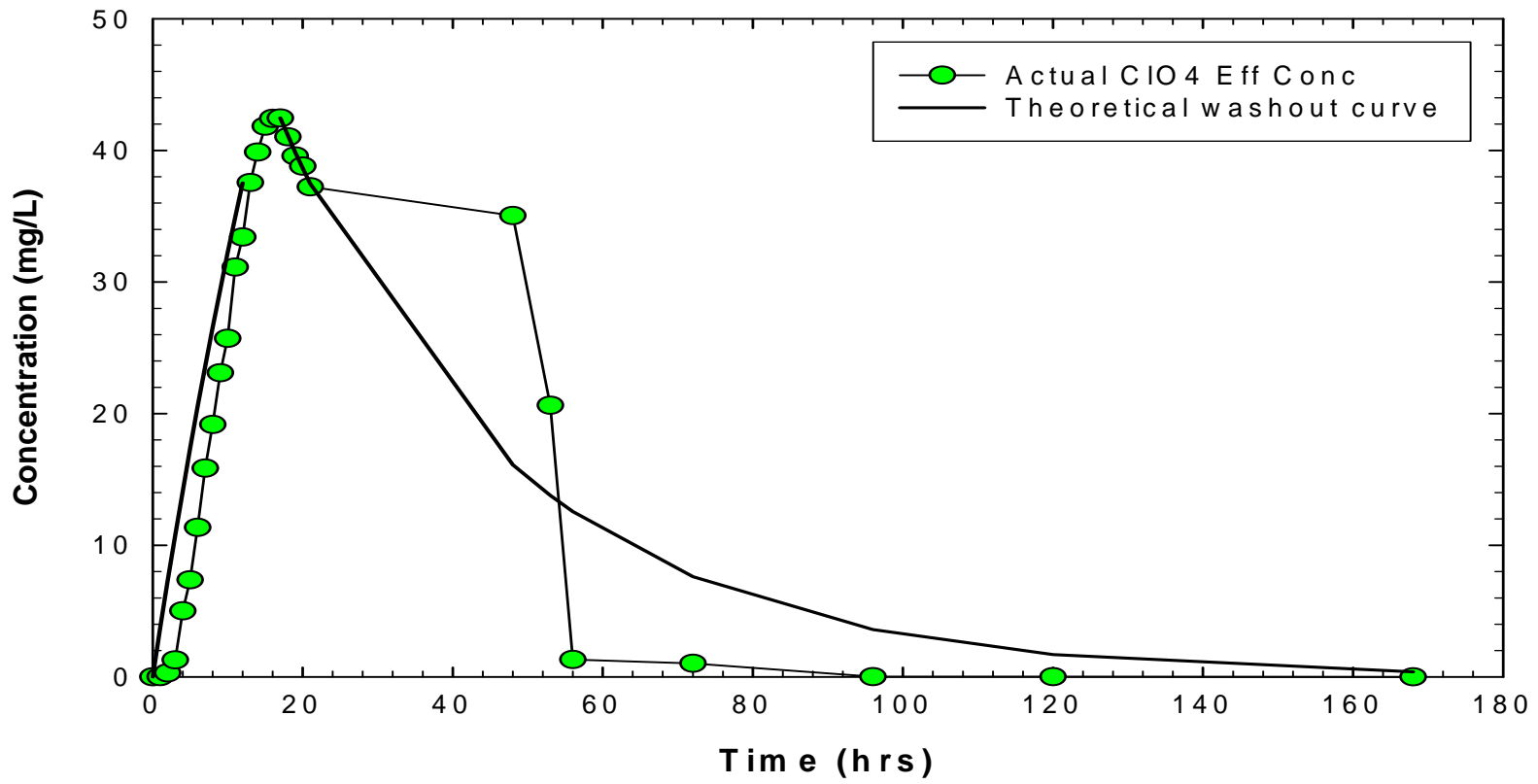


Figure 11. Bioreactor-R comparison of $[\text{ClO}_4^-]$ to the washout curve (second perturbation).

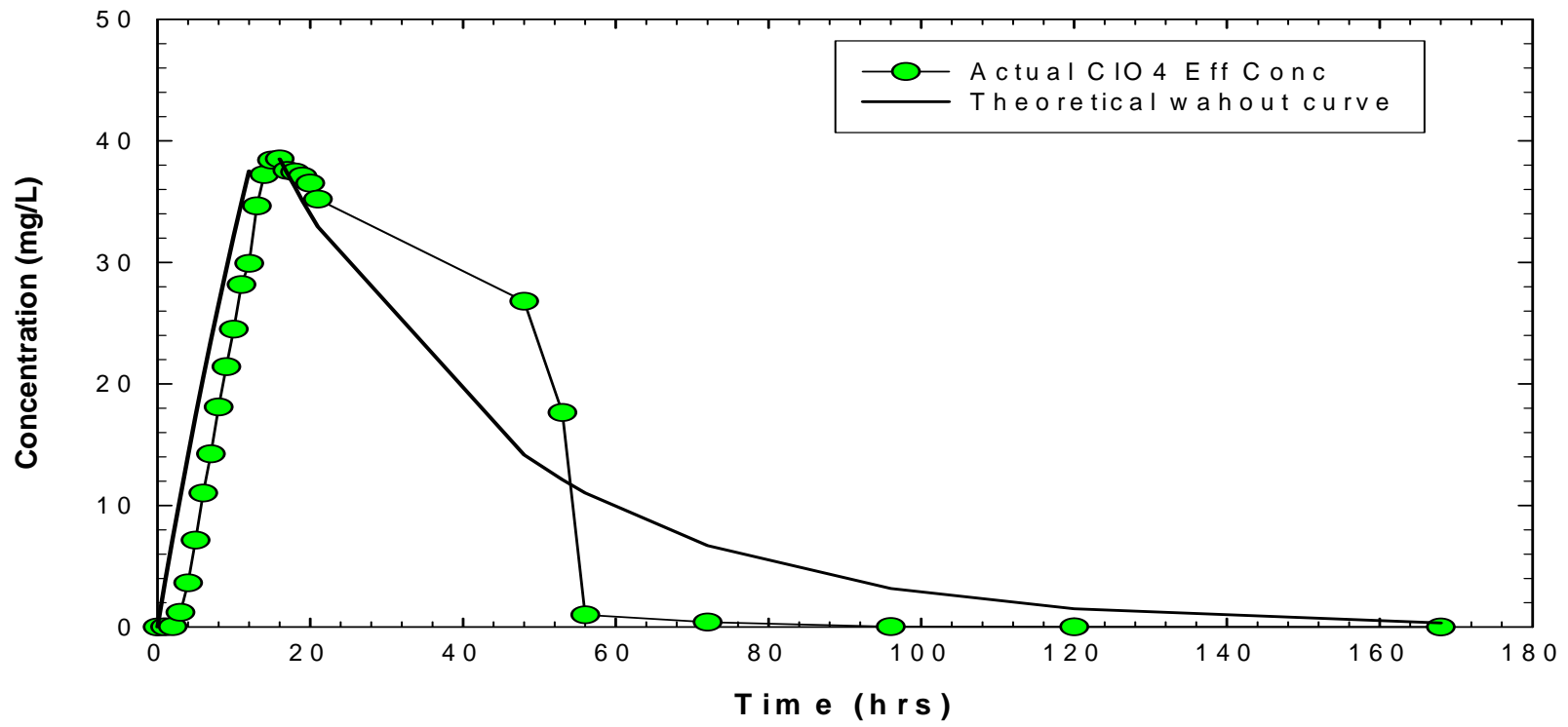


Figure 12. Bioreactor-R comparison of $[\text{ClO}_4^-]$ effluent concentration to the washout curve (third perturbation).

concentration fell below detection level 96 hours after the beginning of the experiments.

It is safe to assume that the microbial population in bioreactor-R was significantly different from those in bioreactor-P and bioreactor-P&R since bioreactor-R was never exposed to perchlorate prior to the initiation of the perturbation experiments. Furthermore, the response of this bioreactor to the second and third feed perturbation suggest that earlier perturbations did not contribute much to acclimation of this bioreactor to the reduction of perchlorate. Dark lines in Figures 10, 11, and 12 represent theoretic accumulation and wash out curves expected if no biodegradation of perchlorate was occurring. The close correspondence between the measured and calculated data suggests that this was indeed the case.

The theoretical accumulation curve was calculated using the following equation:

$$C = 120(1 - e^{-t/32}) \quad (3)$$

where C is effluent perchlorate concentration in mg/L and t is the elapsed time in hours. The 120 represents the influent concentration of perchlorate in mg/L. The equation for the washout curve used is as follows:

$$C = C_A e^{-t/32} \quad (4)$$

where C is effluent concentration, C_A is measured effluent concentration of perchlorate at the peak, and t is time elapsed after the cessation of the introduction of perchlorate in the feed.

As Figures 6–12 show, replication of sampling events was important because it showed a significant difference in stability of microbial communities and their responses to perturbations. It is important to identify the ecological parameters — resistance and resilience — that describe functional stability. Resistance is defined as an immediate response to perturbation, or in the case of this study as a maximum accumulation of the product (Hashsham et al. 2000), while resilience is defined as how fast the system returns to the state before perturbation (Neubert and Caswell 1997). Higher numerical value indicates lower resistance and resilience (Hashsham et al. 2000).

As the above data showed, bioreactor-P, which treated a single contaminant (perchlorate) throughout the entire study, had much lower resistance to toxic shock load than bioreactor-P&R, which treated two contaminants together. In addition, time needed for bioreactor-P to return to the state before perturbation (concentration of perchlorate below detection limit) was much longer compared to bioreactor-P&R. There may be two possible explanations for this kind of behavior: either a pronounced variability exists in the microbial communities of these two bioreactors, or the community structure of these bioreactors may be similar but their responses to perturbations are noticeably different due to the presence of minor populations.

Previous studies (Fernandez et al. 2000; Hashsham et al. 2000) have looked at the response of glucose-fed methanogenic communities to substrate perturbation. Their findings showed that even similar microbial communities can have some minority members, which can greatly impact the response of these systems to substrate perturbation. In addition, they indicated that, even when a single substrate is provided, dynamic microbial populations can develop.

Perturbation experiments in this study involved an electron acceptor instead of the substrate; however, as Figures 6 to 12 illustrate, the presence of minority populations still had a great influence on the response of these bioreactors to perturbations. It is important to note that the perchlorate perturbation did not affect overall performance of the three bioreactors. There was no pH fluctuation, accumulation of volatile fatty acids, or deviations in gas production. In addition, the percent methane in the gas produced in all three bioreactors remained at ~88%, which showed that ethanol was consumed by the microbial community, and that its conversion to CH₄ remained unaffected, even in the absence of perchlorate. A possible electron acceptor that could be used in the absence of perchlorate and would greatly contribute to overall methane production is CO₂, even though most anaerobic bacteria are capable of using variety of electron acceptors including Fe (III), NO₃⁻ and SO₄⁻ (Madigan et al. 2003). It has been reported, however, that the presence of nitrate would decrease perchlorate reduction, due to the fact that some strains of perchlorate respiring bacteria (PRB) are capable of partial or complete denitrification (Xu et al. 2003). In addition, some studies show that SO₄⁻ and Fe (III) cannot be

used as electron acceptors by PRB (Rikken et al. 1996; Coates et al. 1999). This possible shift in electron acceptor can be noticed in bioreactor-P and bioreactor-P&R and can be correlated with their responses to perchlorate perturbations. After the removal of perchlorate from the feed of bioreactor-P, PRB of this reactor possibly shifted their electron acceptor. Uptake of an alternate electron acceptor weakened the perchlorate respiring community, and their response to the first perturbation resulted in much lower resistance and resilience (Figure 6). However, PRB were the most abundant in this bioreactor (since this reactor was treating only perchlorate throughout the entire study); therefore, very little to no substrate competition was occurring, resulting in improved reactor responses to the second and third perturbations (much higher resistance and resilience).

On the other hand, the response of bioreactor-P&R to perturbations was quite the opposite from the response of bioreactor-P. As the data in Figure 7 show, this bioreactor did not respond well to perturbation, probably due to substrate competition among perchlorate respiring and RDX reducing bacteria. Throughout the duration of this study, RDX reducers were out-competed by PRB. This result can be noted from the previous ethanol experiments where it took less time and ethanol concentration to reduce perchlorate to below detectable levels, while RDX remained at the 100 µg/L mark regardless of the magnitude of ethanol concentration in the system. When perchlorate was taken out of the feed during the perturbation experiments, however, PRB remained in the system due to the consumption of an alternate electron acceptor, but their efficiency weakened. This high residual population of PRB may be the reason why the reactor responded well to the first perturbation (high resistance and resilience). Nevertheless, each repetition of the perturbation experiment resulted in a decrease of PRB population, yielding to the lower resistance of the bioreactor to perturbation and a longer recovery time. Due to this result, with each perturbation, there was an abundance of substrate in the system, which allowed for a shift in community structure in favor of RDX reducers. Therefore, when the concentration of ethanol was increased to 550 mg/L during the third perturbation, this abundance of substrate and lower substrate competition (weakened population of PRB) resulted in a slight improvement in RDX reduction (discussed in RDX removal, page 44).

The data in Figures 10, 11, and 12 suggest that bioreactor-R had significantly different microbial community as opposed to bioreactor-P and bioreactor-P&R. Throughout the entire duration of this study, this reactor was treating RDX solely, developing a strong RDX reducing community,

which was exposed to low or no substrate competition. Due to this low exposure, the microbial community of this bioreactor had dramatically low resistance to the shock load of perchlorate during the first perturbation experiment, which would most probably lead to reactor malfunction if perchlorate was not taken out of the feed. However, some biodegradation of perchlorate did occur during the second and third sampling event. These two perturbation experiments showed that the microbial community of this bioreactor possessed a relatively low abundance of PRB, which was outcompeted by the RDX reducers. Nevertheless, change in operational parameters resulted in a slight change in microbial community structure, which led to enhancing the PBR and slight degradation of perchlorate. This reactor showed that the repetitive perturbation can possibly lead toward development of minor populations and populations that are more resilient (Fernandez et al. 2000; Briones and Raskin 2003).

Insufficient data were collected in this study to definitively state that community resistance and change were responsible for the inability of Bioreactor R to degrade perchlorate. This result was unexpected, as other studies involving anaerobic perchlorate removal had not shown a bacterial community unable to degrade perchlorate. More studies designed specifically to address community resistance are needed to definitively answer this question.

DGGE analysis

To discern any differences in the microbial community structure of the three bioreactors, the molecular tool DGGE was used. A gel image of DGGE is shown in Figure 13. The figure contains nine analyses, organized first by reactor (bioreactors-P, -R, or -P&R), and then separated into three lines representing different times of sampling for each reactor. For this analysis, samples were taken during three time intervals, before the first perturbation (line 1), after the first perturbation (line 2), and after the third and final perturbation experiment (line 3). Each band in the DGGE image represents different species. As can be seen from this figure, some bands are more intense than others, which is indicative of predominant species in the system. The strong bands that are common among all three bioreactors are indicated in the image by the solid black arrows. As the band pattern of bioreactor-P shows, differences in the band intensity of the three samples are not significant, indicating no change in microbial community structure of this bioreactor over the three sampling periods. A similar observation can be made about bioreactor-R and bioreactor-P&R. However, line 3 for each reactor shows a lack of a band in the sample

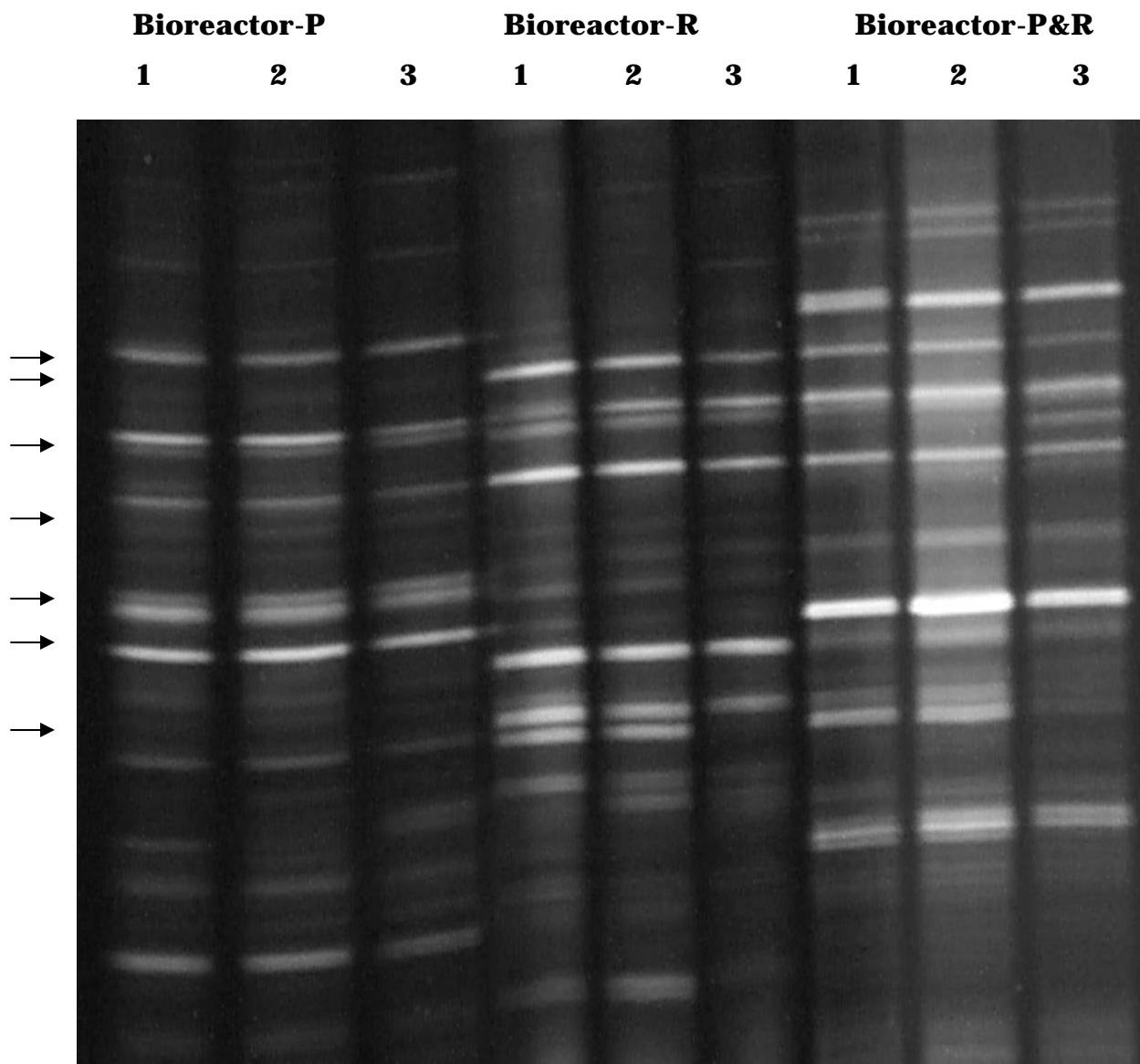


Figure 13. DGGE image of the three bioreactors.

taken after the third perturbation in both bioreactor-R and -P&R (last black solid arrow). The disappearance of this band suggests a possible shift in microbial community structure.

Phylogenetic analysis

A phylogenetic tree of the DGGE gel was constructed using Ward's algorithm (Ward 1963), and the results are shown in Figure 14. As can be seen from the dendrogram, bioreactor-P and bioreactor-R are more closely related to each other than bioreactor-P&R. Despite this fact, the similarity between bioreactor-P and bioreactor-P&R shows that the community structures after perturbation experiments #2 and #3 were more closely

related than the community structure before perturbation #1. This similarity indicates that the shift in microbial community structure occurred in both bioreactors following the perturbation experiments.

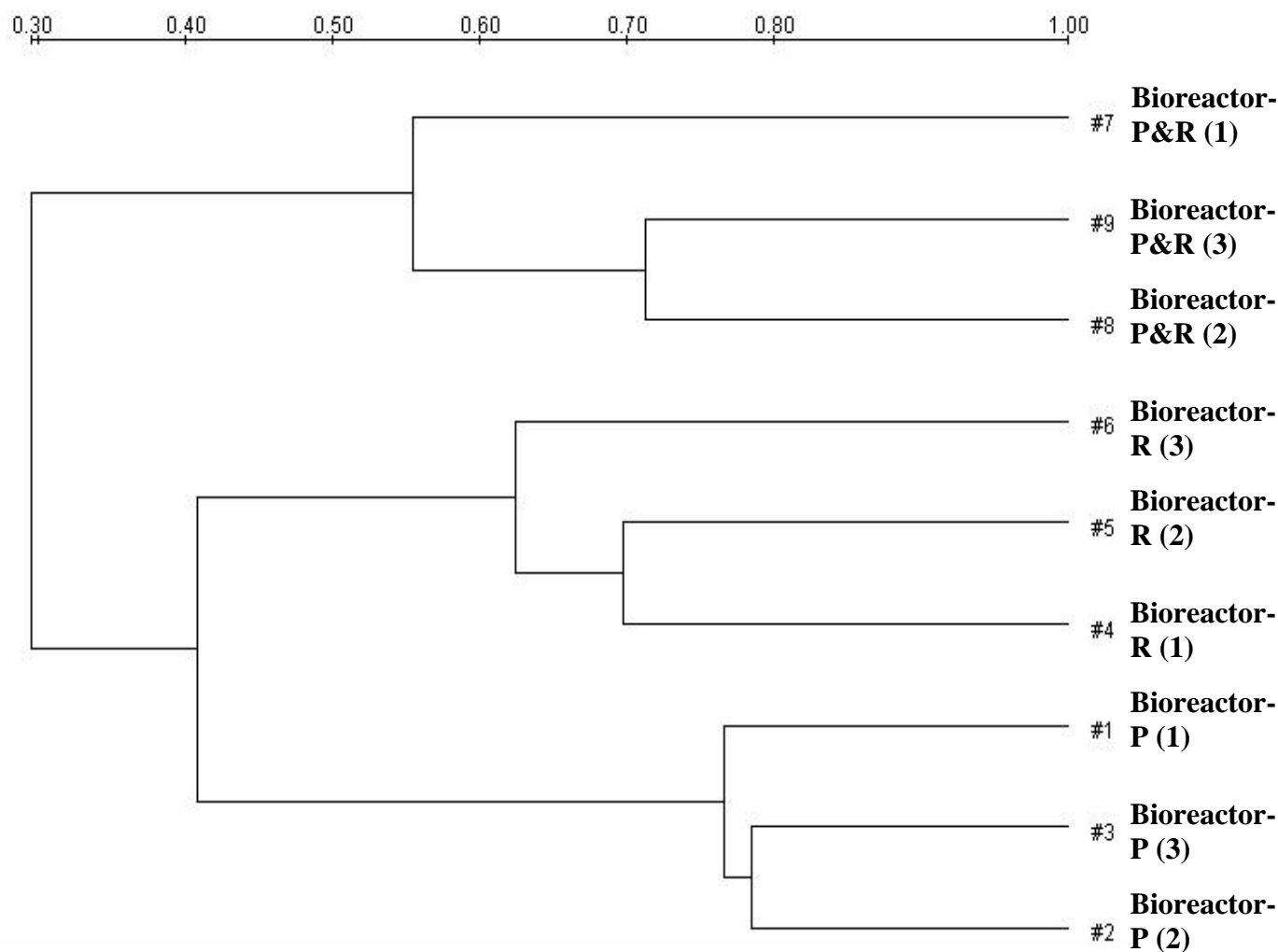


Figure 14. Dendrogram of the DGGE gel.

As stated previously and due to low substrate competition, bioreactor-P exhibited higher resistance and resilience after each perturbation experiment. Therefore, regardless of the clustering difference between the samples taken before the perturbation experiment (#1) and samples taken after (#2 and #3) the shift in microbial community structure of this bioreactor was very low to none. However, perturbation experiments were more detrimental for bioreactor-P&R than to bioreactor-P, due to stronger substrate competition in bioreactor-P&R than in bioreactor-P. This is why the difference between the samples taken before (#1) and after (#2 and #3) the perturbation experiments is more pronounced and the shift in mi-

crobial community structure is more evident in this bioreactor compared to bioreactor-P.

Cluster analysis of bioreactor-R deviates from the previous two bioreactors. It shows that the samples before (#1) and after (#2) the first perturbation experiment are more closely related to each other from the sample taken after the third perturbation (#3). These results closely follow the perturbation experiment results (Figures 10, 11, and 12) where it was shown that, after the first perturbation experiment, perchlorate was completely washed out of the system, while after the second and third perturbation experiments, partial perchlorate degradation may have occurred. Despite the fact that bioreactor-R had a poor response to perturbations, cluster analysis of the dendrogram suggests that the shift in microbial community structure of this bioreactor towards the development of minor and more resilient populations may have occurred.

Sequencing analysis

To see if the three bioreactors share similar community structures, further sequencing of common bands was performed. Comparison of isolated sequences to the National Institute of Health's Basic Local Alignment Search Tool (BLAST) program (<http://www.ncbi.nlm.nih.gov/BLAST/>) did not result in a similarity with any of the cultured bacteria. Isolates were shown as uncultured methanogenic bacteria by the BLAST program, which resulted in the inability to further identify the microbial community of the bioreactors. However, this does not mean that the PBR and RDX reducers were not yet identified. Numerous studies have shown that the perchlorate-respiring bacteria belong to the group of facultative anaerobic gram-negative β -proteobacteria, with the predominant isolates belonging to genera *Dechloromonas* and *Dechlorosoma* (Xu et al. 2003; Wallace et al. 1998; Nozawa-Inoue et al. 2005). In addition, many RDX reducers have been identified as facultative anaerobes belonging to the family of Enterobacteriaceae (*Klebsiella pneumoniae*, *Serratia marcescens*, *Morganella morganii*, *Citrobacter freundii*, and *Escherichia coli*) and several strains of the genus *Clostridium* (Zhao et al. 2003; Adrian and Arnett 2004).

RDX removal

Figures 15 and 16 present the concentration of RDX in the influent and effluent of bioreactor-R and bioreactor-P&R. As can be seen from Figure 15, during the first 100 days of operation of bioreactor-R, the feed concentration of ethanol was set to 50 times the required stoichiometric amount.

During this period, the concentration of RDX in the effluent from this bioreactor gradually decreased from $\sim 800 \mu\text{g/L}$ to $\sim 200 \mu\text{g/L}$.

After steady-state operation was established, the feed ethanol concentration was reduced to 25 times the required stoichiometric amount on day 156. Due to this drop in ethanol, the concentration of RDX increased to $\sim 300 \mu\text{g/L}$, after which concentration of ethanol was raised to 75 times the stoichiometric demand (day 232). On day 274, the concentration of RDX in the feed was reduced from 20 mg/L to 10 mg/L. This resulted in a sharp drop in the concentration of RDX in the effluent from $\sim 200 \mu\text{g/L}$ to below $100 \mu\text{g/L}$.

The bioreactor was run under these ethanol conditions for approximately 100 days with the concentration of RDX remaining at the same level. On day 385, the concentration of electron donor was increased to 200 times its stoichiometric amount with the corresponding concentration of RDX in the final effluent decreasing to below the detection limit. The effluent RDX concentration continued to be below the detection limit even when the feed concentration of ethanol was reduced to 35 times its stoichiometric amount (day 624).

The performance of bioreactor-P&R relative to RDX removal is shown in Figure 16. This graph shows that, during the first 100 days of operation of this reactor, a pronounced variability was observed in effluent RDX concentrations. Nevertheless, during the period extending from days 100 to 156, the effluent RDX concentration started to stabilize in the neighborhood of $500 \mu\text{g/L}$.

On day 156, the influent ethanol concentration was reduced to 2.5 times the stoichiometric demand, which resulted in a gradual decrease of effluent RDX concentration (from 500 to $350 \mu\text{g/L}$). Right after ethanol reduction, the concentration of RDX in the effluent remained at approximately $500 \mu\text{g/L}$, but as the microbial consortia started adapting to the lower electron donor conditions, a gradual drop in the effluent RDX effluent was observed. On day 250, the concentration of RDX in the effluent increased from $\sim 300 \mu\text{g/L}$ to $\sim 1000 \mu\text{g/L}$.

This sharp increase in the effluent RDX concentration corresponded to an error in feed preparation that resulted in an influent RDX concentration of 24 mg/L. On day 274, the concentration of RDX in the influent was

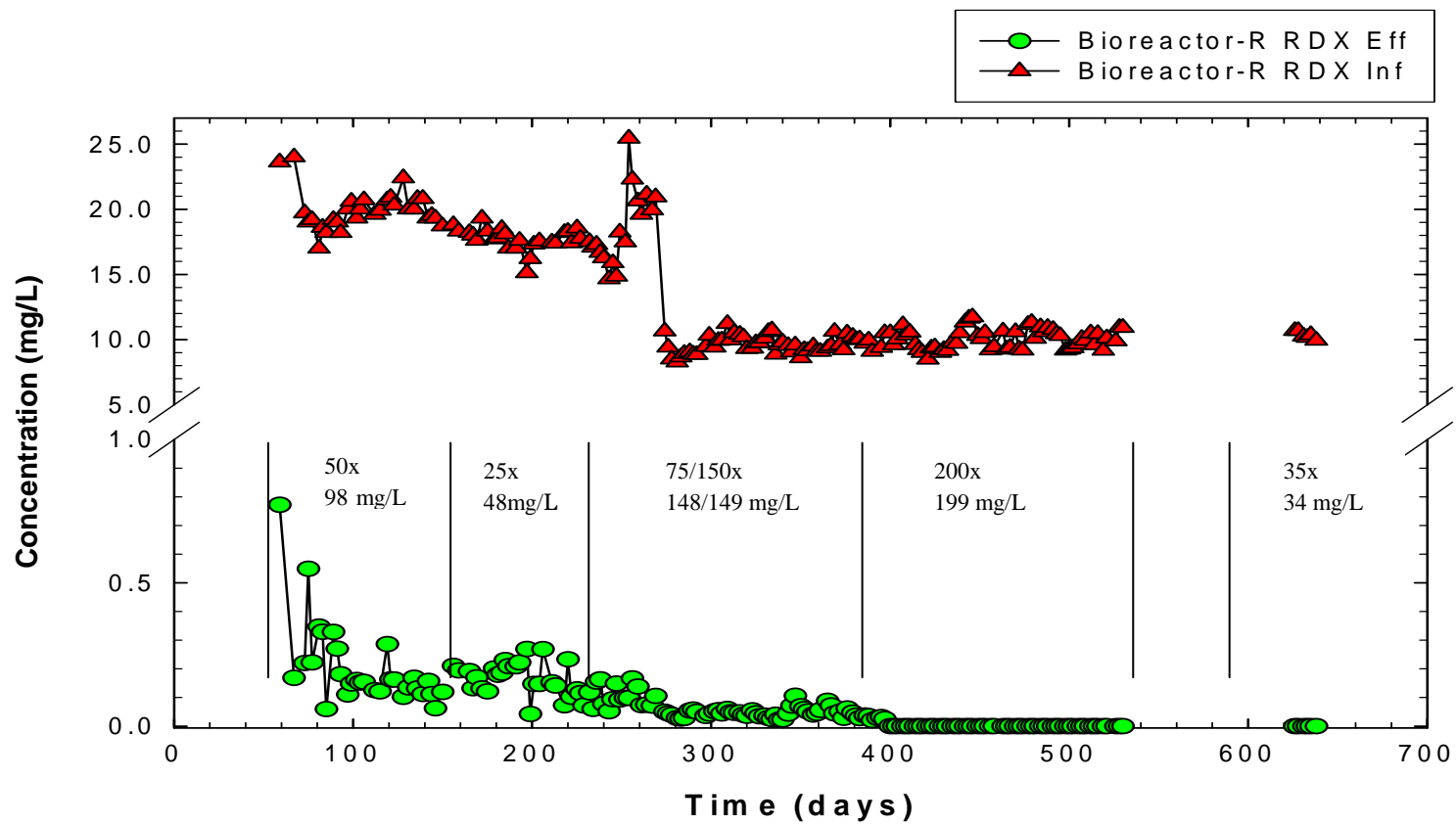


Figure 15. Bioreactor-R RDX reduction prior to perturbations.

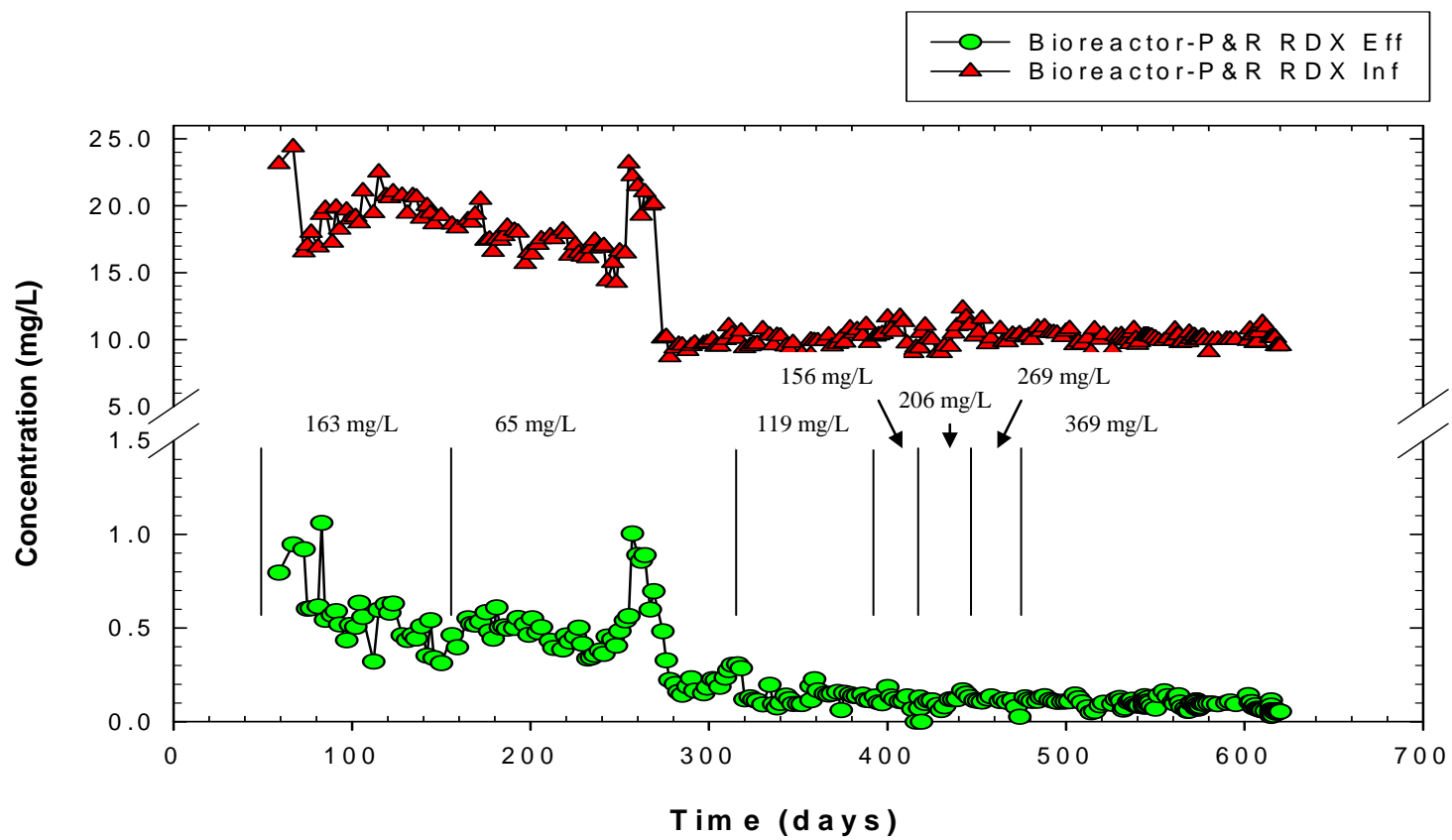


Figure 16. Bioreactor-P&R RDX reduction prior to perturbations.

decreased from the target 20 mg/L to 10 mg/L, which resulted in a decrease in the effluent RDX concentrations to approximately 200 µg/L. When the influent ethanol concentration was increased to 3.9 times its stoichiometric amount (day 319), another drop in the effluent concentration of RDX was observed. Under these conditions, the effluent RDX concentration stabilized at 100 µg/L, and even when the ethanol was raised four more times (to 4.9, 6.23, 7.9 and 10.5 times its stoichiometric amount) the concentration of RDX in the final effluent did not go below this level. Excess ethanol concentrations are represented in Figures 15 and 16 in mg/L.

Perturbation experiments

RDX removal in bioreactor-R corresponding to the period of perturbed perchlorate feeding is represented in Figure 17. As can be seen from this figure, addition of perchlorate to the bioreactor that had never treated this contaminant did not have any discernable effect on RDX removal. Concentrations of RDX remained below detection levels for all three sampling events.

Bioreactor-P&R (Figure 18) exhibited similar behavior in response to the removal of perchlorate from its feed for a 2-week period before its reintroduction. This experiment was repeated three times with no negative impact on RDX removal. During the first experiment, the effluent concentrations of RDX started at approximately 120 µg/L (days 1 and 2), followed by a short period of lower effluent concentrations in the neighborhood of 60 µg/L (days 3 and 4). The concentration of RDX in the final effluent jumped up to about 100 µg/L on day 5, leveling off at approximately 90 µg/L towards the end of the experiment.

Somewhat opposite behavior occurred during the second experiment. The concentration of RDX started at approximately 100 µg/L (days 1 and 2) followed by a slight increase to 140 µg/L (days 3 and 4). After this, concentrations gradually dropped to approximately 80 µg/L (days 6–9) and finally leveled off at 100 µg/L. After the second experiment, the feed concentration of ethanol was increased to 14.5 times its stoichiometric amount (day 591). This rise in ethanol significantly affected performance of the bioreactor during the third experiment. On day 1 of this experiment, the effluent concentration of RDX was approximately 140 µg/L. As the experiment progressed, the effluent concentrations of this contaminant gradually decreased, reaching approximately 80 µg/L towards the end of the sampling event.

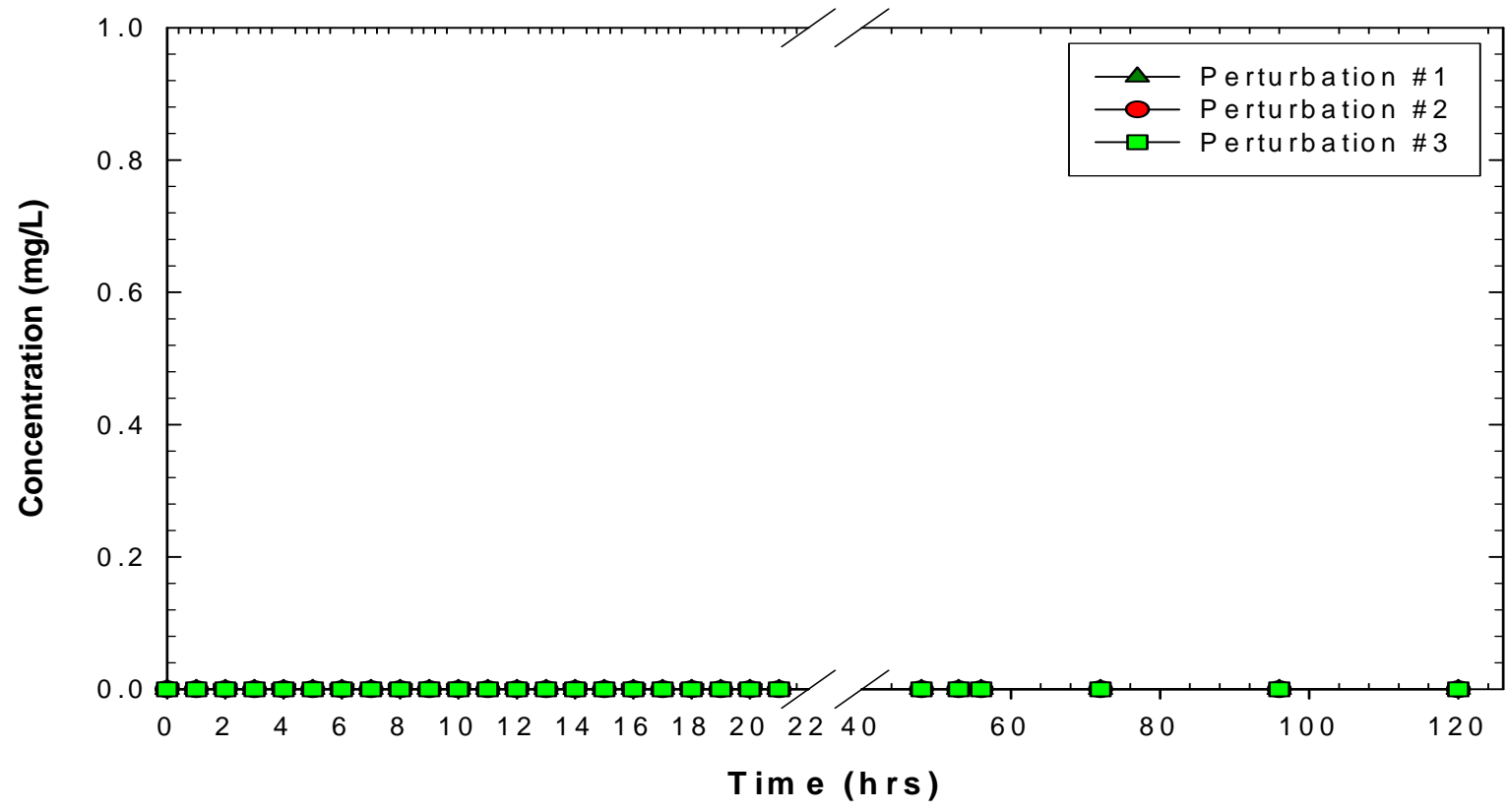


Figure 17. Bioreactor-R RDX reduction during perturbations.

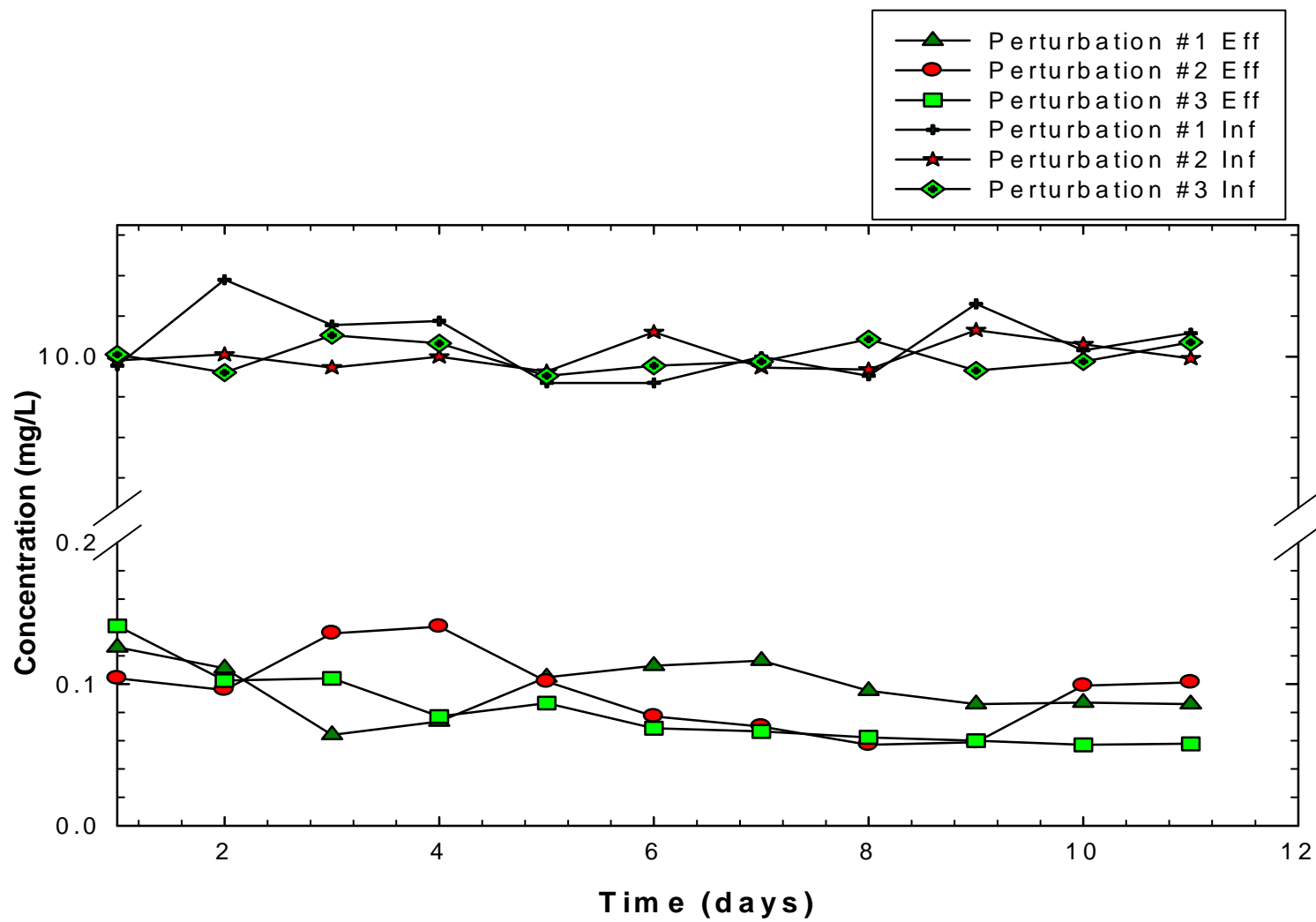


Figure 18. Bioreactor-P&R RDX reduction during periods without perchlorate (2 weeks no ClO₄⁻ in the feed).

These data show that short-term removal of perchlorate from the bioreactor did not allow RDX to be degraded to below detection levels. However, the data do demonstrate that the presence of perchlorate exerts a strong effect on microbial selection based on the inability of Bioreactor R to degrade perchlorate.

The response of bioreactor-P&R to reintroduction of perchlorate into the feed is represented in Figure 19. This figure clearly suggests a marked improvement in RDX removal during the third feed perturbation experiment. As mentioned before, this improvement in reactor response after the third perturbation is probably due to the abundance of the substrate and the low substrate competition among the two groups of microorganisms. The response of bioreactor-P&R remained relatively unchanged during the first and second experiments.

Volatile fatty acids analysis

The concentrations of volatile fatty acids (VFAs) in the effluent from all three bioreactors are shown in Tables 5, 6, and 7. As can be seen from these data, the effluent from bioreactor-P contained only acetic acid, while the effluent from bioreactor-R contained both acetic and propionic acid. The effluent from bioreactor-P&R contained the three major VFAs: acetic, propionic, and butyric acids. Pivalic acid was used as an internal standard during the measurement of VFAs.

Data from Table 5 (bioreactor-P) show relatively low effluent concentrations of acetic acid from day 51 to day 155 (average value of 1.59 mg/L). As the concentration of ethanol in the feed was decreased to 92.5 mg/L, the concentration of acetic acid decreased to 0.03 mg/L. After further reduction in ethanol feed concentration to 46.25 mg/L (days 319 to 332), the concentration of this VFA went below the detection limit. Increase in the influent ethanol concentration back to 92.5 mg/L (days 333 to 384) did not result in an increase of the VFA concentrations. Only when the concentration of electron donor was decreased to 69 mg/L did the concentration of acetic acid increase to 1.01 mg/L.

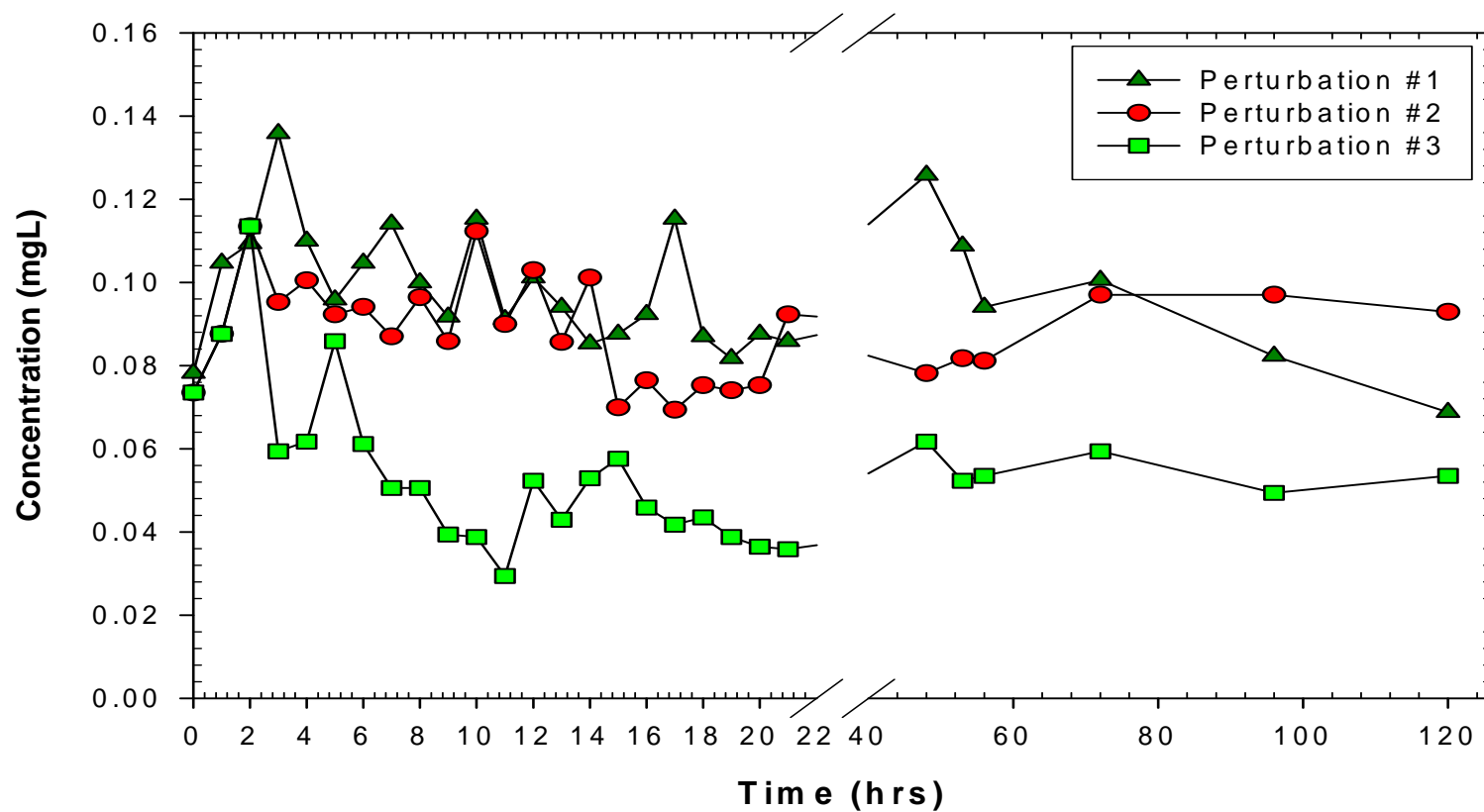


Figure 19. Bioreactor-P&R RDX reduction during perturbations (after perchlorate reintroduced).

Table 5. Bioreactor-P volatile fatty acids concentrations.

Period	Ethanol Feed Conc. (mg/L)	Acetic Acid (mg/L)	Propionic Acid (mg/L)	n-Butyric Acid (mg/L)	i-Butyric Acid (mg/L)	Valeric Acid (mg/L)
52-155	185.00	1.59 (± 0.47)	-	-	-	-
156-318 333-384	92.50	0.03 (± 0.13)	-	-	-	-
319-332	46.25	0.00 (± 0.00)	-	-	-	-
385-714	69.37	1.01 (± 1.54)	-	-	-	-

Table 6. Bioreactor-R volatile fatty acids concentrations.

Period	Ethanol Feed Conc. (mg/L)	Acetic Acid (mg/L)	Propionic Acid (mg/L)	n-Butyric Acid (mg/L)	i-Butyric Acid (mg/L)	Valeric Acid (mg/L)
52-155	100.00	41.69 (± 3.85)	27.00 (± 3.39)	-	-	-
156-231	50.00	19.67 (± 5.28)	9.06 (± 3.71)	-	-	-
232-384	150.00	37.94 (± 17.25)	16.26 (± 9.66)	-	-	-
385 - 532	200.00	4.24 (± 2.10)	1.25 (± 0.65)	-	-	-
533 - 623	400.00	1.42 (± 2.02)	0.54 (± 0.94)	-	-	-
624 - 714	35.00	0.85 (± 0.12)	0.49 (± 0.03)	0.27 (± 0.02)	0.37 (± 0.04)	-

As can be seen from Table 6 (bioreactor-R), the effluent concentrations of acetic and propionic acid during the period from day 52 to day 155 was relatively high, with concentrations averaging 41.69 mg/L and 27.00 mg/L, respectively. As the influent ethanol concentration was decreased from 100 mg/L to 50 mg/L (days 156 to 231), the effluent concentrations of the two acids decreased to average values of 19.67 mg/L for acetic acid and 9.06 mg/L for propionic acid. An increase in the influent concentration of ethanol to 150 mg/L (days 232 – 384), however, resulted in higher effluent concentrations of these two VFAs (37.94 mg/L of acetic acid and 16.26 mg/L of propionic acid). When the feed ethanol concentration was increased to 200 mg/L (days 385 to 532), the concentration of acetic and propionic acid decreased to 4.24 mg/L and 1.25 mg/L, respectively.

Table 7. Bioreactor-P&R volatile fatty acids concentrations.

Period	Ethanol Feed Conc. (mg/L)	Acetic Acid (mg/L)	Propionic Acid (mg/L)	n-Butyric Acid (mg/L)	i-Butyric Acid (mg/L)	Valeric Acid (mg/L)
52 – 155	195.00	59.76 (± 20.00)	16.28 (± 6.76)	0.43 (± 0.20)	-	-
156 – 318	97.50	12.29 (± 3.67)	-	-	-	-
319 – 384	150.00	8.37 (± 4.51)	0.34 (± 0.44)	-	-	-
385 – 414	187.00	5.94 (± 0.97)	1.69 (± 0.50)	-	-	-
415 – 447	237.00	9.52 (± 4.79)	4.26 (± 3.18)	-	-	-
448 – 471	300.00	12.01 (± 3.64)	5.82 (± 1.52)	-	-	-
472 – 590	400.00	6.37 (± 5.35)	2.87 (± 2.83)	-	-	-
591 – 714	550.00	2.07 (± 2.59)	1.00 (± 1.70)	-	-	-

Further increase in ethanol concentrations to 400 mg/L (days 533 to 623) resulted in even lower concentrations of the two VFAs (average values of 1.42 mg/L of acetic acid and 0.54 mg/L of propionic acid). Subsequent reduction in the influent ethanol concentration to 35 mg/L (day 624) resulted in lower concentrations of acetic and propionic acid (0.85 mg/L and 0.49 mg/L, respectively); nevertheless, traces of n-butyric and i-butyric acid appeared at this time (0.27 mg/L and 0.37 mg/L, respectively).

Data from Table 17 (bioreactor-P&R) shows rather high concentrations of acetic and propionic acid and a trace of butyric acid (days 52 to 155), but, when the ethanol concentration was reduced to 97.5 mg/L (days 156–318), the concentration of acetic acid decreased drastically to 12 mg/L, while propionic and butyric acid could not be detected in the effluent any more. However, a further increase in the influent concentration of electron donor to 150 mg/L (days 319–384) did not result in a corresponding increase of the concentration of VFAs (acetic acid concentrations averaging 8.37 mg/L).

A relatively small concentration of propionic acid was detected again in the system (average value of 0.34 mg/L). As the concentration of ethanol was increased to 187 mg/L (days 385–414), the concentration of acetic acid in the system continued to drop (average value of 5.94 mg/L) while the con-

centration of propionic acid slightly increased to 1.69 mg/L. When the influent ethanol concentration was raised to 237 mg/L (days 415–447) and 300 mg/L (days 448–471), concentrations of acetic acid increased to 9.52 mg/L and 12.01 mg/L respectively (concentrations of propionic acid increased accordingly to 4.26 and 5.82 mg/L).

In the period between day 472 and day 590, ethanol concentration in the feed was raised to 400 mg/L, which resulted in the decrease of acetic and propionic acid in the system (6.37 mg/L acetic acid and 2.87 mg/L propionic acid). Similarly, when the concentration of ethanol was increased to 550 mg/L the concentration of acetic acid dropped to 2.07 mg/L, while propionic acid measured 1.00 mg/L.

The VFA data presented above suggest no strong correlation between the VFA content of the reactor effluent and the influent concentration of electron donor. In fact, these data suggest that a minimum critical concentration of electron donor was needed to establish stable methanogenic conditions and that, once these conditions are established, all three bioreactors affected good effluent quality relative to the concentration of VFAs.

Gas analysis

The composition of gas produced by the microbial consortia of all three bioreactors is presented in Table 8. As can be seen from the table, the majority of the gas produced is in the form of methane (CH_4), while oxygen (O_2) was detected in very small concentrations, indicative of sampling leaks. In addition, high concentrations of methane, especially in bioreactor-R and -P&R, indicate a low or no presence of RDX in the system, considering that high concentrations of RDX inhibit methane production when ethanol is used as the electron donor (Adrian et al. 2003).

Table 8. Gas analysis.

Gas Composition	Bioreactor-P (%)	Bioreactor-R (%)	Bioreactor-P&R (%)
CO_2	3.10 (± 1.67)	3.41 (± 0.94)	3.11 (± 1.54)
O_2	0.13 (± 0.67)	0.27 (± 8.63)	0.25 (± 2.06)
N_2	8.30 (± 4.99)	8.13 (± 16.64)	8.51 (± 8.36)
CH_4	88.39 (± 4.25)	88.18 (± 17.76)	88.12 (± 10.10)

Oxidation-reduction potential analysis

Measurements of ORP are presented in Table 9. As the table shows, all ORP measurements were negative, indicating reducing conditions in the bioreactors. In addition, measurements in all three sampling events were relatively similar, suggesting that the system perturbations did not have a large impact on the reducing species of the bioreactors. In prior research, Jung et al. (2004) attempted to demonstrate the effectiveness of bioremediation for cleaning ordnance and explosives scrap. What they observed was that, for reduction of explosives such as TNT, an ORP lower than -250 mV was required. The data from their study are in agreement with data obtained in this study, where the ORP measurements also remained below -250 mV.

Table 9. Oxidation-reduction potential measurements.

Period	Bioreactor-P ORP (mV)	Bioreactor-R ORP (mV)	Bioreactor-P&R ORP (mV)
Day 543	-297.40	-274.00	-284.00
Day 572	-301.00	-283.00	-289.10
Day 614	-304.20	-286.70	-291.30

Demonstration-scale (bioreactor-D) perchlorate results

Demonstration-scale tests were conducted in campaigns, as previously described. The length of the campaign tests was limited by the ability to store the wastewater. This limitation was due to a precaution taken in the demonstration-scale tests in which the goal was to have zero discharge of perchlorate into the industrial waste treatment plant. Thus, all of the wastewater was collected and treated by recycling it through the GAC-FBR until the perchlorate level was below the detection limit ($< 1 \mu\text{g/L}$).

Bioreactor-D was a field operating system being used for treatment of TNT and RDX in pinkwater. The operating characteristics were similar to the laboratory tests as shown in Table 4, but analytical capability for the suite of parameters monitored in the laboratory studies was not available. The primary data available are influent and effluent concentrations for explosives (TNT and RDX) and perchlorate.

Tests were conducted with an increasing concentration of perchlorate (from campaign to campaign) until the system appeared to have difficulty degrading the perchlorate. Although a constant influent concentration of

perchlorate was intended for the tests, several factors caused variations in the measured concentration. These include:

1. Wastewater feed rate: The wastewater feed rate was controlled by automatically adjusting a flow valve based on feedback from flow rate measurement. Flow would actually vary by 10% from the set point, so even if the injection were perfectly constant, the influent concentration varied by the differences in overall influent flow to the feed line.
2. System pressure: The wastewater feed was pumped from a float-controlled influent tank. The valve to the feed tank would open at the low point and close when filled. This process resulted in back pressure to the perchlorate injection point that was not constant.
3. Perchlorate reservoir level: As the level of concentrated perchlorate solution dropped in the reservoir (a 10-L polyethylene barrel), the amount injected would decline slightly.

All of these effects combined to produce an uneven concentration of perchlorate in the feedwater. Experiments are referred to as the calculated perchlorate concentration, but the actual concentration was measured after the injected perchlorate solution had mixed with the feedwater. The laboratory-scale reactors (bioreactor-P, -R, and -P&R) cannot be directly compared to the demonstration-scale reactor (bioreactor-D) because the laboratory-scale reactor had a nonadsorptive media (sand) as the biological support, whereas the demonstration-scale system had GAC as the biological support media. Perchlorate is not expected to be adsorbed to any significant extent, however, so comparisons of the perchlorate removal by biological degradation should be valid.

Figure 20 shows the first test conducted at a relatively high concentration of perchlorate on the demonstration-scale plant. Problems were experienced with the influent pump, leading to wide variations of the influent concentration. The target influent concentration was 35 mg/L.

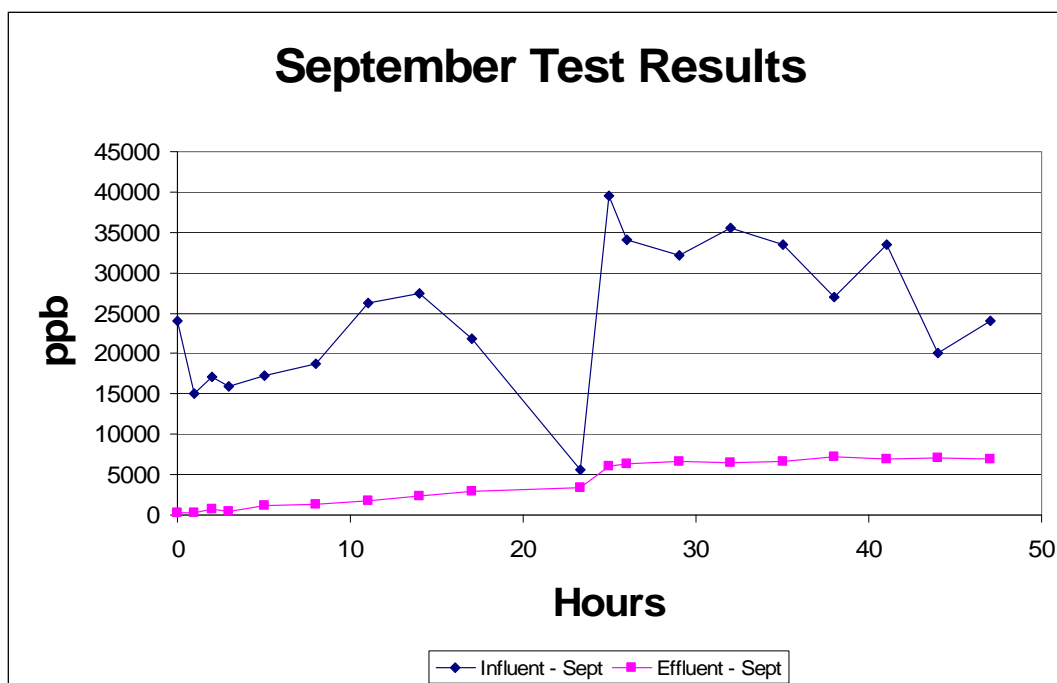


Figure 20. Bioreactor-D at 35 mg/L feed, 2 gpm (38.5 g/m³-day).

The results in Figure 20 indicate that relatively high concentrations of perchlorate passed through the reactor. Effluent concentrations of 7 to 8 mg/L would not be acceptable for discharge from the plant regardless of downstream dilution and potential degradation. Thus, a series of follow-on experiments were conducted in successive months starting with lower concentrations.

Figures 21 to 23 show the follow-on experiments conducted on the demonstration-scale plant. All tests were conducted at a flowrate of 2 gpm.

These initial tests, starting at a relatively low concentration and increasing, showed great promise. The graphs below indicate almost complete removal of perchlorate. A closer look shows that each test had an initial breakthrough, which had only a short duration. This can be seen on Figure 24, which compares effluent only.

The effluent results of the follow-on experiments show that the bacterial populations at the demonstration-scale plant were capable of removing perchlorate to low levels. The levels below 50 ppb may be acceptable for the industrial discharge of GAC-FBR, because it represents a low flow-high strength source that is mixed with other wastewaters before entering the wastewater treatment plant at MCAAP.

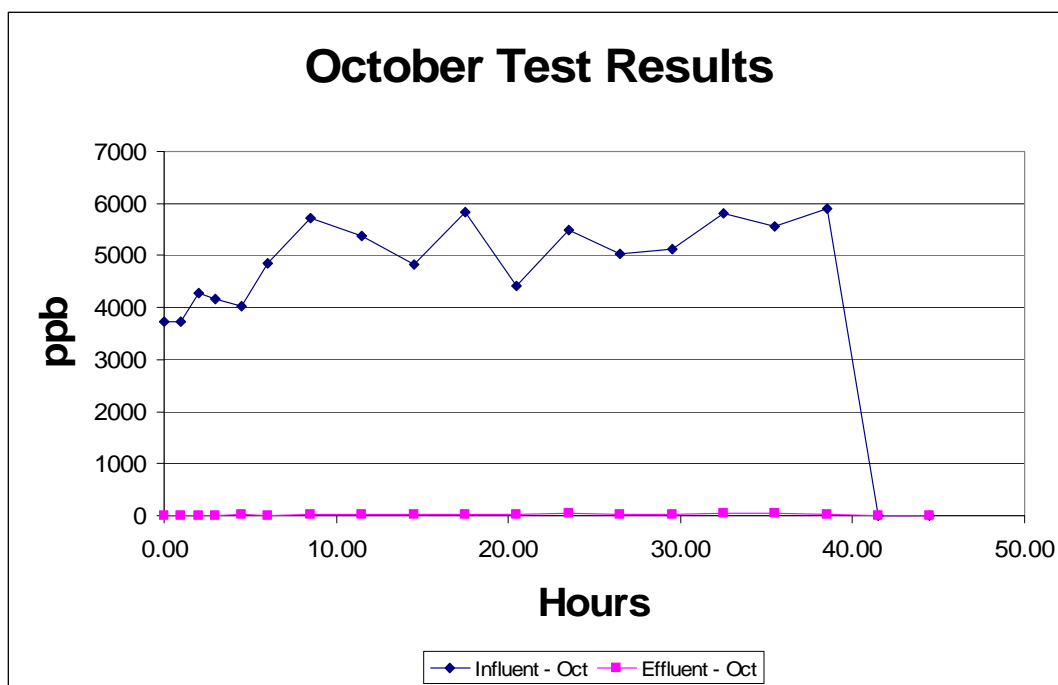


Figure 21. Bioreactor-D at 5 mg/L feed, 2 gpm (5.5 g/m³-day).

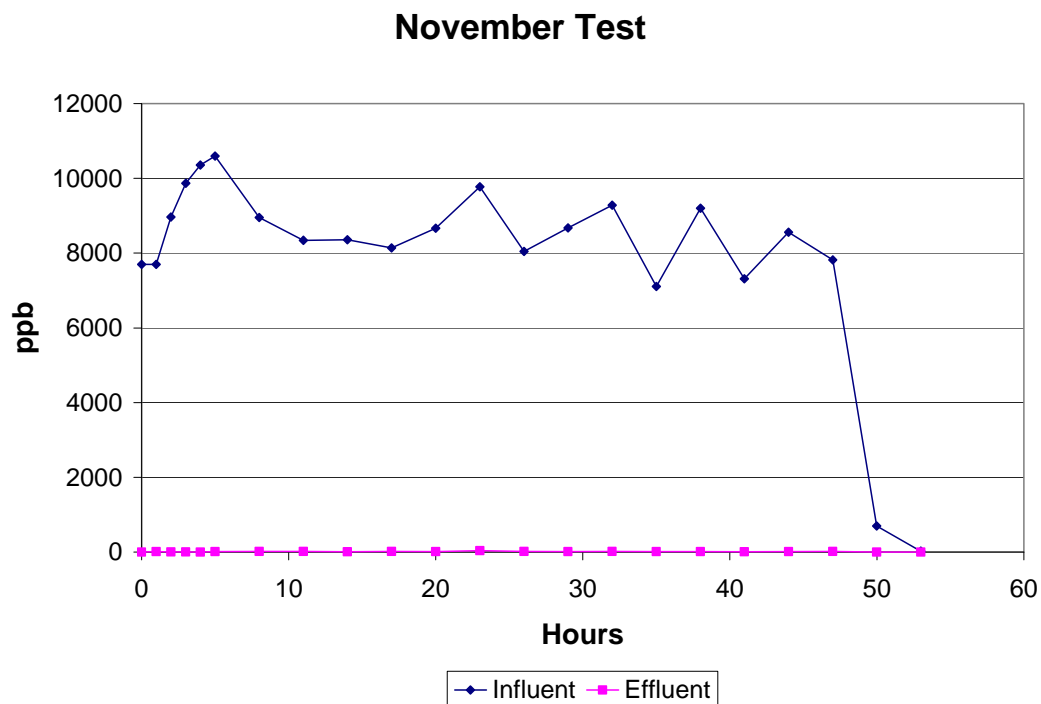


Figure 22. Bioreactor-D at 8 mg/L feed, 2 gpm (8.8 g/m³-day).

December Test

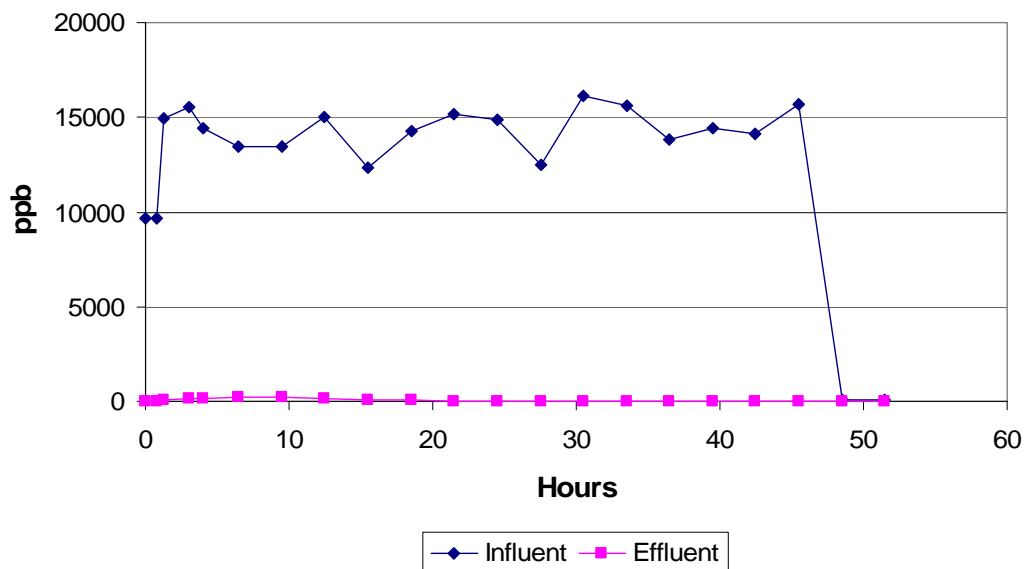


Figure 23. Bioreactor-D at 15 mg/L feed, 2 gpm (16.5 g/m³-day).

Selected Effluents

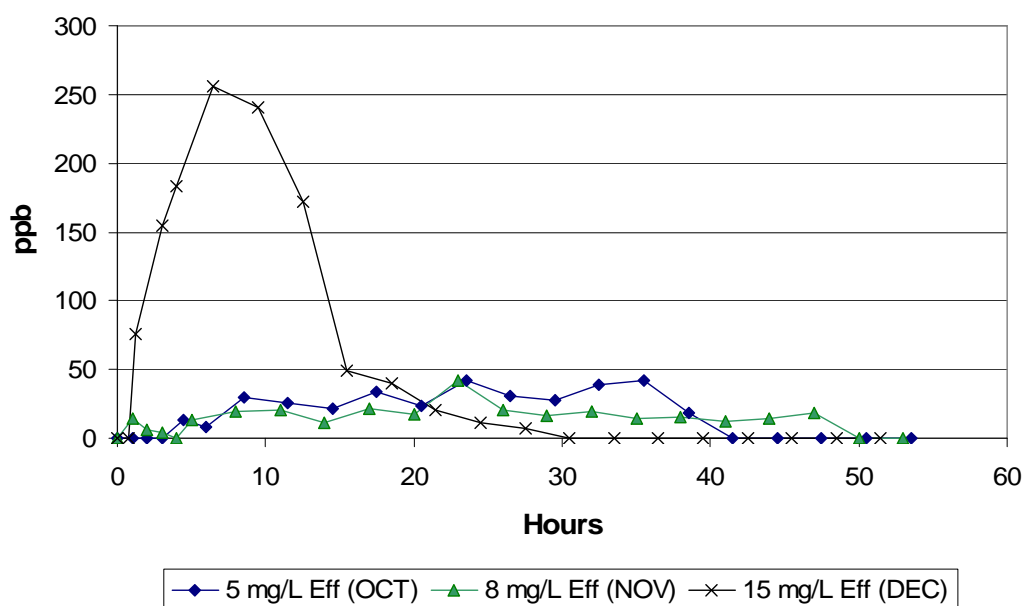


Figure 24. Effluents of October-December 2004 tests on bioreactor-D.

These experiments were conducted in relative short order and had followed the initial test at 35 mg/L, which had poor results. The results suggest that some type of acclimation had occurred, and that the bacterial population had retained that trait. In addition, the last campaign test in this series, conducted at the highest concentration and loading, had shown the best treatment, with effluent concentrations below detection limit after only 30 hours.

Additional tests were performed at 15 mg/L and 2 gpm influent flow rate to further evaluate the effect of acclimation time. This translates to a loading rate of 16.5 g/m³-day. These tests were conducted in February, April, and May 2005. The February tests were conducted at the end of the month, so essentially 2 1/2 months had passed since the December 2004 tests. The April and May tests were conducted in rapid succession, with only 2 weeks between the tests. The objective of the April and May tests was to determine whether a capability remained to degrade perchlorate after a short period.

Figure 25 summarizes the results of all of the tests. Although this figure is very busy, the main point is that the influents of all the tests were all about 15 mg/L, but that considerable variation occurred in the influent during the February tests (due to problems with the injection pump).

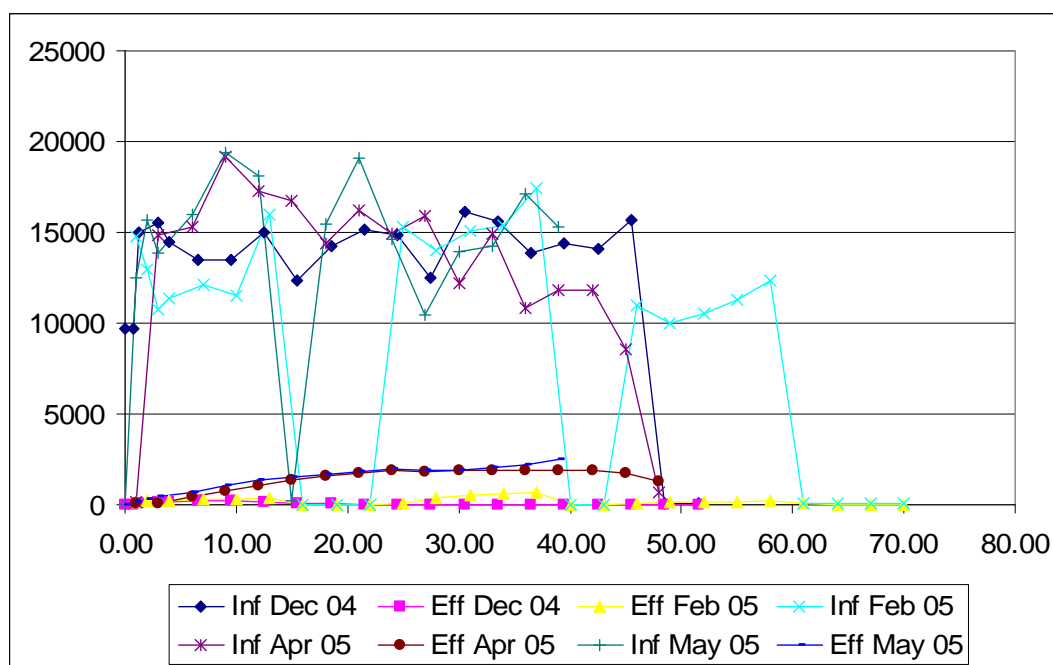


Figure 25. Summary of 15 mg/L tests, 2 gpm, FY2005 (16.5 g/m³-day).

The effluent results can be more easily seen in Figure 26 for the four tests conducted at 15 mg/L influent concentration in fiscal year 2005 (FY2005). The results were not as promising as the earlier set in which the influent concentration of perchlorate was gradually increased from 5 to 15 mg/L at 2 gpm (Figure 24 on previous page).

The results show that the best performance obtained occurred after the gradual increase. The next best performance occurred when pump problems caused the perchlorate to disappear from the influent at two times during the February tests, but even in that case, the effluent reach levels above 700 ppb.

The last two tests were conducted in rapid succession, with only 2 weeks between the campaigns. After the campaign test in April, the system was put in recycle to remove all of the perchlorate, and then started again of pinkwater. The results show, however, that the difference between the two tests was small, except that the April test appeared to have reached a peak in effluent concentration and started to decline.

At this point, the campaign tests were suspended and the perturbation experiments were added to the laboratory-scale studies. As previously discussed, the laboratory-scale reactor, which had received only RDX, demonstrated little ability to degrade perchlorate (see Figures 10–12,

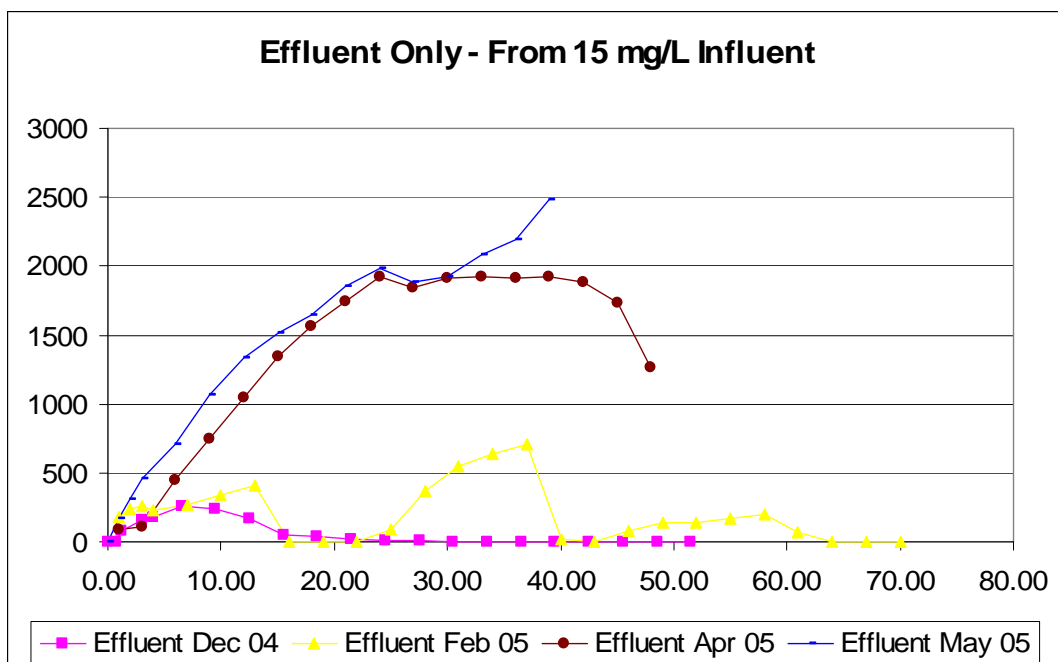


Figure 26. Effluent concentrations from 15 mg/L tests, 2 gpm (16.5 g/m³-day).

bioreactor-R) even while maintaining anaerobic conditions. Conversely, the perchlorate did not seem to degrade the ability of the biomass in bioreactor-R to remove RDX.

After this result was obtained at the laboratory-scale level, three more campaign tests were conducted. The first test was conducted in July 2006 using a higher concentration of perchlorate and lower flowrate (1 gpm), so that the loading to the bioreactor would be the same as for 15 mg/L at 2 gpm. In addition, a comparison of the influent and effluent concentrations was calculated as if there was no degradation of the perchlorate in the reactor. The calculation was based on the assumption that the bioreactor could be modeled as a completely mixed reactor. This assumption is based on the fact that the recycle flowrate on the bioreactor was set at 180 gpm, while the influent flow rate was only 1 gpm.

The general form of this calculation is $\{(\text{mass in}) - (\text{mass out})\} / \text{reactor volume}$. For purposes of this calculation, the average influent concentration during the campaign test was used. Using increments of 1 minute, the following equation was used:

$$\text{Eff} = [\text{Inf}] * Q_i - [\text{Recycle}] * Q_e / \text{Vol}_r + [\text{Recycle}] \quad (5)$$

where:

[Inf] = Influent Concentration as measured in the influent line

[Recycle] = Recycle Concentration from previous iteration

Q_i = Influent Flowrate

Q_e = Effluent Flowrate (which in this case is the same as influent)

Vol_r = Reactor Volume

Figure 27 presents the data for the test in July, in which a target concentration of 30 mg/L was injected at a flowrate of 1 gpm, for a mass loading of 16.5 g/m³-day. The actual average concentration was found to be 31.9 mg/L.

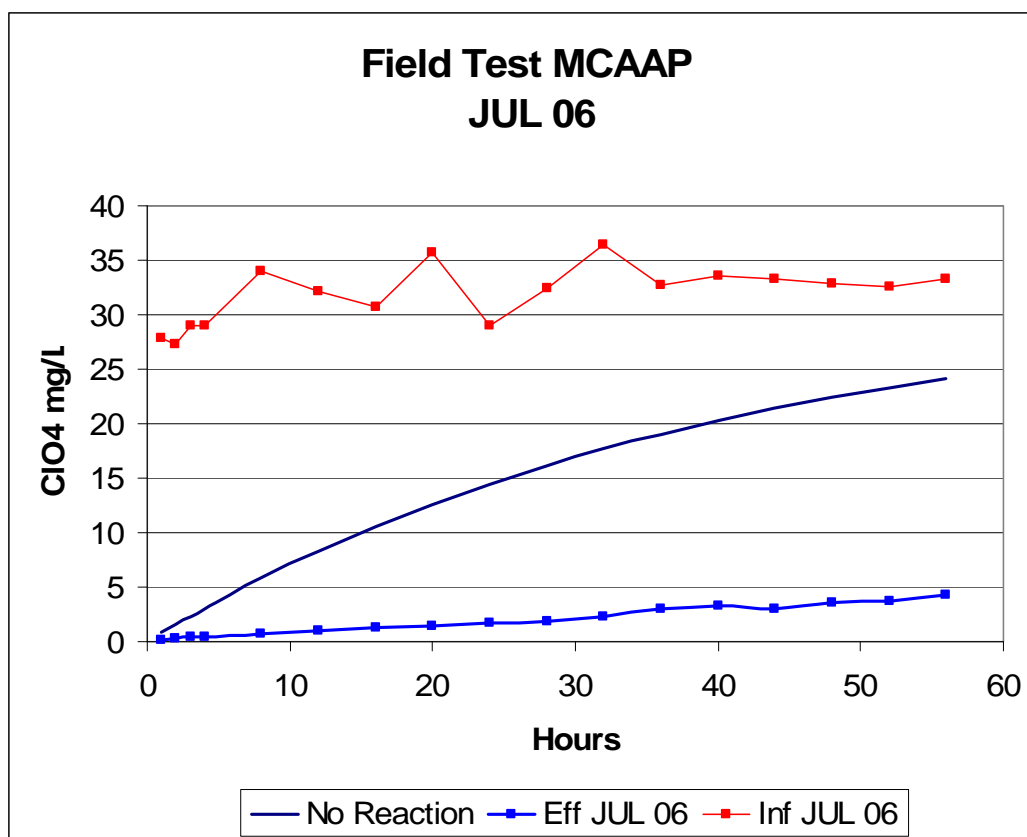


Figure 27. July 2006 test conducted at 30 mg/L, 1 gpm (16.5 g/m³-day).

Comparison of the effluent and calculated concentrations, based on no reaction of perchlorate in the reactor, indicates that significant removal was occurring although the effluent did continue to rise to high levels, even higher than previously observed at this mass loading rate. The bioreactor had not been exposed to perchlorate at this point for several months.

Two additional campaign tests were conducted, at declining loadings, to determine if there was a threshold loading to which the bioreactor could acclimate in a short period. These tests were conducted in August and September 2006, at target perchlorate concentrations of 16 mg/L and 11 mg/L respectively. Figures 28 and 29 show the results obtained, in addition to the calculated perchlorate build-up based on no removal in the bioreactor.

These two data sets show that the bioreactor continues to remove the majority of the perchlorate applied (~ 80%) in each case, but does not get to a point where perchlorate-degrading bacteria are sufficient to remove perchlorate to below detection limits in 3 days or fewer.

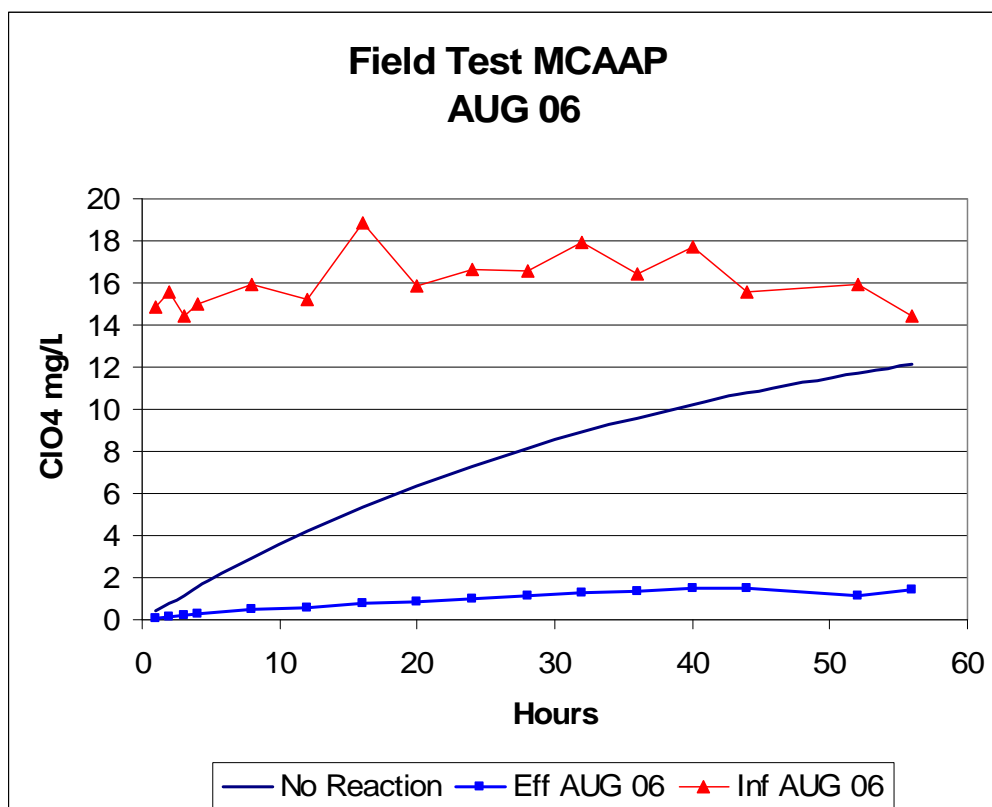


Figure 28. August 2006 test conducted at 16 mg/L, 1 gpm (8.8 g/m³-day).

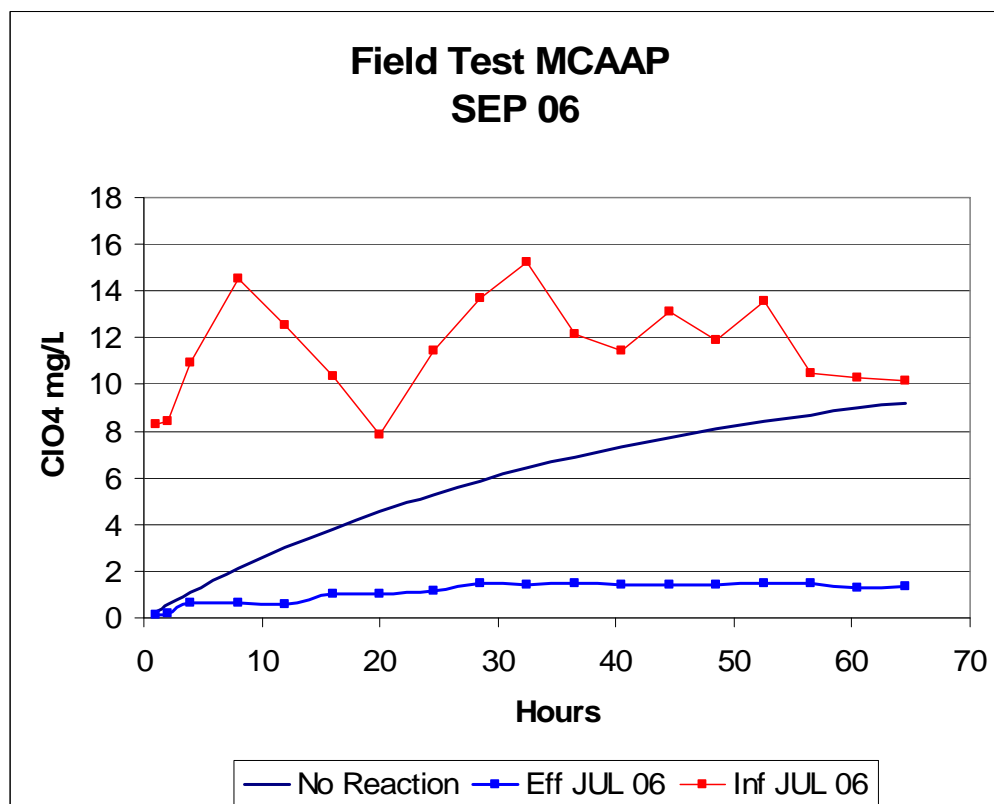


Figure 29. September 2006 test conducted at 11 mg/L, 1 gpm (6.1 g/m³-day)

Tests conducted over the entire campaign series at MCAAP show that perchlorate-reducing bacteria are always present, but that their numbers vary greatly. The first series of tests, for which Figure 24 summarizes their effluent, seemed to indicate that perchlorate-reducing bacteria were sufficient to degrade the perchlorate to below detection limits in a very short period and that only a very short acclimation time would be needed (< 2 days). All of the subsequent tests at the demonstration-scale showed that, although perchlorate-reducing bacteria were present, they were not found in sufficient numbers to degrade the perchlorate to below detection limits in one pass through the bioreactor.

This result differs from the laboratory-scale results, where bioreactor-R had essentially no capability to degrade perchlorate when the perturbation tests began. The main difference is that bioreactor-R (and all laboratory-scale systems) was fed a well-controlled synthetic wastewater, whereas the demonstration-scale bioreactor was treating actual wastewater. Although the main contaminants in that wastewater were TNT and RDX, the water was collected from washdown of industrial operations and stored in open systems, so a much more diverse population of bacteria was constantly being introduced to the influent.

Regardless of the ability to degrade perchlorate in one pass through the demonstration bioreactor, recycling the effluent allowed the biomass in that reactor to eventually remove perchlorate to below detection limits in all cases. Thus, there is no limiting concentration below which the biomass in the bioreactor could not effectively treat. Therefore, for small batches of perchlorate-bearing wastewater, the bioreactor is effective in removing perchlorate to below detection limits.

5 Conclusions

Perchlorate and RDX are two toxic compounds used extensively by the U.S. military. Their use has led to leakage of the compounds into the environment. A recent development in munition design has led to the use of these materials in combination, which has never been done before. The current method for removal of RDX from wastewater is carbon adsorption, but carbon adsorption would be ineffective against a polar compound such as perchlorate.

The most common method for removal of perchlorate is biodegradation, but anaerobic biodegradation of RDX is still an emerging technology and has never been tested in combination with perchlorate at the high concentrations expected in wastewater. This study has shown that biological treatment with AFBRs is a good alternative for effective removal of RDX and perchlorate from wastewater. Results from this study showed that the biodegradation of perchlorate and RDX depends on the concentrations of electron donor (ethanol) added to the bioreactors. Perchlorate seemed to require a lower dosage of electron donor in order to be reduced from the high influent concentration levels used in this study. However, higher concentrations of ethanol (relative to stoichiometric requirements) were needed to achieve effective reduction of RDX. Nevertheless, biodegradation seemed to be greater in the bioreactors containing individual contaminants rather than in the bioreactor where both contaminants were commingled.

Perturbation experiments were performed in order to test the resistance and resilience of the microbial community structure in the three bioreactors. What these experiments suggest is that the competition of microbial consortia for the availability of the substrate played a major role in structure and function of that community. The perturbation experiments did not involve the disturbance of substrate; rather they involved the disruption of the electron acceptor. However, shifts in microbial community structure were observed. The bioreactor that had an abundance of the substrate (bioreactor-P) recovered rather fast after each perturbation and its resistance and resilience significantly improved by the end of the third perturbation experiment. On the other hand, the bioreactor that was exposed to strong substrate competition (bioreactor-P&R) exhibited a shift in microbial community structure that occurred after disruption in the

feed of the electron acceptor. This resulted in a decrease in resistance and resilience after each perturbation. Lastly, the bioreactor exposed to RDX only (bioreactor R) showed that anaerobic populations can be selected over long experimental periods in which there is little or no capability to use perchlorate as an electron acceptor. Further, this particular anaerobic population was not disturbed by the presence of high concentrations of perchlorate.

Campaign tests conducted on bioreactor-D were similar to the perturbation tests on the laboratory-scale reactors. However, the campaign tests represent a combination of the three laboratory-scale reactors. Perchlorate did not appear to interfere with the anaerobic degradation of explosives, but the demonstration-scale bioreactor could not remove high concentrations of perchlorate upon initial exposure. Prolonged exposure (but always less than 1 week) through recycling allowed bioreactor-D to remove perchlorate to below detection limits in all cases. This result indicates that a period of acclimation is necessary, and that period is greater than 4 days. Initial work in this bench study required 200 days to reach steady removal of perchlorate to below detection limits. A separate laboratory-scale study (Veenstra et al. 2003; Weeks et al. 2003; Schaefer et al. 2007) at low concentrations in groundwater required approximately 50 days to achieve steady removal of perchlorate to below detection limits. In both cases, once steady removal was achieved, it was maintained. In the case of the bench study reported on here, the biomass was shown to easily recover after short (2-week) periods without perchlorate in the feed.

The demonstration-scale results were not as clear. The demonstration-scale bioreactor, which was rarely exposed to perchlorate, was always able to remove 80% of the perchlorate on a single pass, but was not able to remove perchlorate to below the detection limit within 2.5 days (the length of the experimental run was limited by the ability to store effluent for recycling).

The demonstration-scale unit is shown to be capable of removing perchlorate to below detection limits as long as the effluent can be recycled. At MCAAP this means, in practical terms, as long as the wastewater generated is less than the effluent tank storage capacity (10 kgal). Flow-through treatment of high concentrations has been shown to be feasible in the laboratory-scale tests reported on here, and has been shown to be feasible for low concentrations typical of groundwater in other field applications. Sporadic appearance of perchlorate at high concentrations in the influent

may be a problem, and it is recommended that a continuous feed of perchlorate-contaminated wastewater be maintained, either through use of a large influent basin or through feeding small concentrations to the influent stream to maintain the capability of the biomass to degrade perchlorate.

References

- Adrian, N. R., and C. M. Arnett. 2004. Anaerobic Biodegradation of Hexahydro-1,3,5-Trinitro-1,3,5-Triazine (RDX) by *Acetobacterium malicum* Strain HAAP-1 Isolated from a Methanogenic Mixed Culture. *Current Microbiology*. **48**, 332-340.
- Adrian, N. R., C. M. Arnett, and R. F. Hickey. 2003. Stimulating the Anaerobic Biodegradation of Explosives by the Addition of Hydrogen or Electron Donors that Produce Hydrogen. *Water Research*. **37**, 3499-3507.
- Briones, A., and L. Raskin. 2003. Diversity and Dynamics of Microbial Communities in Engineered Environments and their Implications for Process Stability. *Current Opinions in Biotechnology*. **14**, 270-276.
- Coates, J. D., and L. A. Achenbach. 2004. Microbial Perchlorate Reduction: Rocket-Fuelled Metabolism. *Nature Reviews*. **2**, 569-580.
- Coates, J. D., U. Michaelidou, A. B. Royce, S. M. O'Connor, J. N. Crespi, and L. A. Achenbach. 1999. Ubiquity and Diversity of Dissimilatory (Per)chlorate Reducing Bacteria. *Applied and Environmental Microbiology*. **65**(12), 5234-5241.
- Coleman, M. L., M. Ader, S. Chaudhuri, and J. D. Coates. 2003. Microbial Isotopic Fractionation of Perchlorate Chlorine. *Applied and Environmental Microbiology*. **69**, 4997-5000.
- De Luca, L. L., F. Cozzi, and G. Germinias. 1999. Combustion Mechanisms of an RDX-Based Composite Propellant. *Combustion and Flame*. **118**, 248-261.
- Environmental Security Technology Certification Program (ESTCP). 2000. Ammonium Perchlorate Biodegradation for Industrial Wastewater Treatment. Department of Defense, ESTCP Program Office, Arlington, VA.
<http://www.estcp.org/Technology/upload/199710.pdf>
- _____. 2003. Mineralization of TNT, RDX and their By-Products in an Anaerobic Granular Activated Carbon-Fluidized Bed Reactor. Department of Defense, ESTCP Program Office, Arlington, VA.
<http://www.estcp.org/documents/techdocs/200004.pdf>
- EPA Method 314.0. 1999. Determination of Perchlorate in Drinking Water Using Ion Chromatography, Revision 1. U.S. Environmental Protection Agency.
- EPA Method 8330. 1994. Nitroaromatics and Nitramines by High Performance Liquid Chromatography (HPLC). U.S. Environmental Protection Agency.
- Fernandez, A.S., S. A. Hashsham, S. L. Dollhopf, L. Raskin, O. Glagoleva, F. B. Dazzo, R. F. Hickey, C. S. Criddle, and J.M. Tiedje. 2000. Flexible Community Structure Correlates with Stable Community Function in Methanogenic Bioreactor Communities Perturbed by Glucose. *Applied and Environmental Microbiology*. **66**(9), 4058-4067.

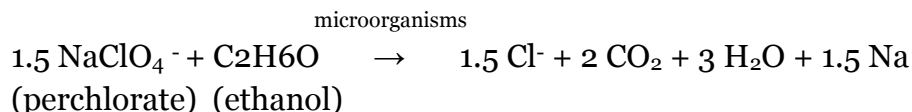
- Fuller, M. E., P. B. Hatzinger, C. W. Condee, and A. P. Togni. 2007. Combined treatment of perchlorate and RDX in Groundwater Using a Fluidized Bed Reactor. *Groundwater Monitoring and Remediation*. **27**(3), 59-64.
- Grace, J. R., and H. Bi. 1997. Introduction to Circulating Fluidized Beds. In Grace, J. R., Avidan, A. A., and Knowlton, T. M. (Eds) *Circulating Fluidized Beds*. Blackie Academic and Professional, London, UK.
- Gupta, C. K., and D. Sathiyamoorthy. 1999. Fluid Bed Technology in Materials Processing. CRC Press, Boca Raton, FL, 1-7 and 472-477.
- Halasz, A., J. Spain, L. Pacquet, C. Beaulieu, and J. Hawari. 2002. Insights into the Formation and Degradation Mechanisms of Methylenedinitramine During the Incubation of RDX with Anaerobic Sludge. *Environmental Science and Technology*. **36**(4), 633-638.
- Hashsham, S. A., A. S. Fernandez, S. L. Dollhopf, F. B. Dazzo, R. F. Hickey, J. M. Tiedje, and C. S. Criddle. 2000. Parallel Processing of Substrate Correlates with Greater Functional Stability in Methanogenic Bioreactor Communities Perturbed by Glucose. *Applied and Environmental Microbiology*. **66**(9), 4050-4057.
- Hatzinger, P. A. 2005. Perchlorate Biodegradation for Water Treatment, *Environmental Science and Technology*, **39**(11), 239A-247A.
- Hautman, D. P., D. J. Munch, A. D. Eaton, and A. W. Haghani. 1999. EPA Method 314.0 Determination of Perchlorate in Drinking Water Using Ion Chromatography.
- Hawari, J., A. Halasz, T. Sheremata, S. Beaudet, C. Groom, L. Paquet, C. Rhofir, G. Ampleman, and A. Thiboutot. 2000. Characterization of Metabolites During Biodegradation of Hexahydro-1,3,5-Trinitro-1,3,5-Triazine (RDX) with Municipal Anaerobic Sludge. *Applied and Environmental Microbiology*. **66**, 2652-2657.
- Jung, C. M., D. A. Newcombe, D. L. Crawford, and R. L. Crawford. 2004. Detection and Decontamination of Residual Energetics from Ordnance and Explosives Scrap. *Bioremediation*. **15**, 41-48.
- Madigan, M. T., J. M. Martinko, and J. Parker. 2003. Brock Biology of Microorganisms. Prentice Hall, New Jersey, 576-587.
- Maloney, S. W., E. G. Engbert, M. T. Suidan, and R. F. Hickey. 1998. Anaerobic Fluidized Bed Treatment of Propellant Wastewater. *Water Environment Research*. **70**(1), 52-59.
- Maloney, S. W., N. R. Adrian, R. F. Hickey, and R. L. Heine. 2002. Anaerobic Treatment of Pinkwater in a Fluidized Bed Reactor Containing GAC. *Journal of Hazardous Materials*. **92**, 77-88.
- McCormick, N. G., J. H. Cornell, and A. M. Kaplan. 1981. Biodegradation of Hexahydro-1,3,5-Trinitro-1,3,5-Triazine. *Applied and Environmental Microbiology*. **42**, 817-823.
- Motzer, W. E. 2001. Perchlorate: Problems, Detection, and Solutions. *Environmental Forensics*, **2**, 301-311.

- Muyzer, G. 1999. Genetic fingerprinting of microbial communities-present status and future perspectives. Proceedings of the 8th International Symposium on Microbial Ecology. C. R. Bell, M. Brylinsky, P. Johnson-Green (eds). Atlantic Canada Society for Microbial Ecology, Canada. Microbial Biosystems: New Frontiers.
- Muyzer, G., and K. Smalla. 1998. Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. *Antonie van Leeuwenhoek*. **73**, 127-141.
- Muyzer, G., E. C. De Waal, and A. G. Uitterlinden. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Applied Environmental Microbiology*. **59**, 695-700.
- Muyzer, G., T. Brinkhoff, U. Nubel, C. Santegoeds, H. Schafer, and C. Wawer. 2004. Denaturing Gradient Gel Electrophoresis (DGGE) in Microbial Ecology. Microbial Ecology Manual, 2nd ed. Kluwer Academic/Plenum Publisher, The Netherlands.
- Neubert, M. G., and H. Caswell. 1997. Alternatives to Resilience for Measuring the Response of Ecological Systems to Perturbations. *Ecology*. **78**(3), 653-665.
- Nozawa-Inoue, M., K. M. Scow, and D. E. Rolston. 2005. Reduction of Perchlorate and Nitrate by Microbial Communities in Vadose Soil. *Applied and Environmental Microbiology*. **71**(7), 3928-3934.
- Polk, J., C. Onewokae, W. J. Guarini, C. Murray, D. E. Tolbert, and A. P. Tonga. 2002. Army Success Story: Ex-situ Biological Treatment of Perchlorate-Contaminated Groundwater, *Federal Facilities Environmental Journal*, Summer Edition, 85-94.
- Rikken, G. B., A. G. Kroon, and C. G. van Ginkel. 1996. Transformation of (Per)chlorate into Chloride by a Newly Isolated Bacterium: Reduction and Dismutation. *Applied Microbiology and Biotechnology*. **45**, 420-426.
- Schaefer, C. E., M. E. Fuller, C. W. Condee, J. M. Lowey, and P. B. Hatzinger. 2007. Comparison of biotic and abiotic treatment approaches for co-mingled perchlorate, nitrate, and nitramine explosives in groundwater. *Journal of Contaminant Hydrology*. **89**, 231-250.
- Song, Y., and B. E. Logan. 2004. Effect of O₂ Exposure on Perchlorate Reduction by *Dechlorosoma* sp. KJ. *Water Research*. **38**, 1626-1632.
- Suidan, M. T., J. T. Pfeffer, and G. F. Nakhala. 1998. Anaerobic Expanded-Bed GAC Reactor for the Treatment of Biologically Inhibiting Wastes Generated During Coal and Petroleum Distillation. In Anaerobic Digestion 1988: Proceedings of the 5th International Symposium on Anaerobic Digestion held in Bologna, Italy, 22-26 May, 1998. Pergamon Press, New York, NY.
- Urbansky, E. T., and M. R. Schock. 1999. Issues in Managing the Risk Associated with Perchlorate in Drinking Water. *Journal of Environmental Management* **56**, 79-95.
- Urbansky, E.T. 2000. Perchlorate in the Environment. Kluwer Academic/Plenum Publisher, New York, NY, 15-29 and 241-256.

- Van Aken, B. V., J. M. Yoon, and J. L. Schnoor. 2004. Biodegradation of Nitro-Substituted Explosives 2,4,6-Trinitrotoluene, Hexahydro-1,3,5-Trinitro-1,3,5-Triazine, and Octahydro-1,3,5,7-Tetranitro-1,3,5-Tetrazocine by a Phytosymbiotic *Methylobacterium* sp. Associated with Poplar Tissues (*Populus deltoids* x *nigra* DN34). *Applied and Environmental Microbiology*. **70**, 508-517.
- VanderLoop, S. L., M. T. Suidan, M. A. Moteleb, and S. W. Maloney. 1999. Biotransformation of 2,4-dinitrotoluene under different electron acceptor conditions. *Water Research*, **33**(5), 1287-1295.
- Veenstra, S. C., K. R. Weeks, D. L. Hill, and A. P. Tonga. 2003. Streamlined Treatment Option for Remediation of Commingled Perchlorate and Explosives in Groundwater, presented at the National Defense Industrial Association 29th Environmental and Energy Symposium and Exhibition, April, Richmond, VA.
- Wallace, W., S. Beshear, D. Williams, S. Hospadar, and M. Owens. 1998. Perchlorate Reduction by a Mixed Culturer in an Up-flow Anaerobic Fixed Bed Reactor. *Journal of Industrial Microbiology and Biotechnology*. **20**, 126-131.
- Wang, Y. T., M. T. Suidan, and B. E. Rittmann. 1987. Modeling Biofilm Kinetics for a Low-Loaded Expanded-Bed Anaerobic Reactor. *Biotechnology and Bioengineering*, **XXX**, 5-21.
- Ward, J. H. 1963. Hierarchical grouping to optimize an objective function. *Journal of American Statistical Association*. **58**, 236-244.
- Weeks, K. R., S. C. Veenstra, D. L. Hill, and B. P. Gregson. 2003. A Study of Treatment Options to Remediate Explosives and Perchlorate in Soils and Groundwater at Camp Edwards, Massachusetts. *Remediation*. Spring 2003, 131-143.
- Xu, J., Y. Song, B. Min, L. Steinberg, and B. E. Logan. 2003. Microbial Degradation of Perchlorate: Principles and Applications. *Environmental Engineering Science*. **20**(5), 405-422.
- Zhao, J.S., J. Spain, and J. Hawari. 2003. Phylogenetic and Metabolic Diversity of Hexahydro-1,3,5-Trinitro-1,3,5-Triazine (RDX)-Transforming Bacteria in Strictly Anaerobic Mixed Cultures Enriched on RDX as Nitrogen Source. *FEMS Microbiology Ecology*. **46**, 189-196.

Appendix A: Stoichiometric Ethanol Requirements for AFBRs

Bioreactor-P



$$\text{MW}_{(\text{ClO}_4^-)} = 99.5 \text{ g/L}$$

$$\text{MW}_{(\text{C}_2\text{H}_6\text{O})} = 46 \text{ g/L}$$

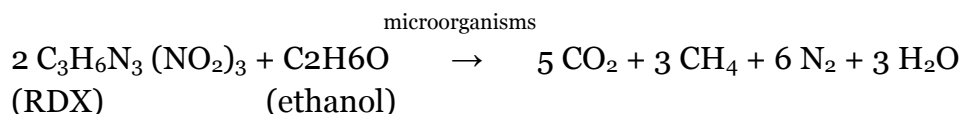
Target concentration of perchlorate in the final feed = 120 mg/L

$$\text{Formula: } 1.5(\text{MW}_{(\text{ClO}_4^-)}) / 1(\text{MW}_{(\text{C}_2\text{H}_6\text{O})}) = \text{g/L ClO}_4^- / X$$

$$\text{Calculation: } 1.5 (99.5 \text{ g/L}) / (46 \text{ g/L}) = 0.12 \text{ g/L} / X$$

$$\mathbf{X = 0.0369 \text{ g/L} = 36.9 \text{ mg/L}}$$

Bioreactor-R



$$\text{MW}_{(\text{C}_3\text{H}_6\text{N}_3(\text{NO}_2)_3)} = 222 \text{ g/L}$$

$$\text{MW}_{(\text{C}_2\text{H}_6\text{O})} = 46 \text{ g/L}$$

Target concentration of RDX in the final feed = 20/10 mg/L

$$\text{Formula: } 2 (\text{MW}_{(\text{C}_3\text{H}_6\text{N}_3(\text{NO}_2)_3)}) / 1(\text{MW}_{(\text{C}_2\text{H}_6\text{O})}) = \text{g/L RDX} / X$$

$$\text{Calculation: } 2 (222 \text{ g/L}) / (46 \text{ g/L}) = 0.02 \text{ g/L} / X$$

$$\mathbf{X = 0.002 \text{ g/L} = 2 \text{ mg/L}}$$

$$2 (222 \text{ g/L}) / (46 \text{ g/L}) = 0.01 \text{ g/L} / X$$

$$\mathbf{X = 0.001 \text{ g/L} = 1 \text{ mg/L}}$$

Bioreactor-P&R

$$36.9 \text{ mg/L} + 2 \text{ mg/L} = 38.9 \text{ mg/L}$$

$$36.9 \text{ mg/L} - 1 \text{ mg/L} = 35.9 \text{ mg/L}$$

Appendix B: Bioreactor Effluent Quality Data

Table A1. Bioreactor-P effluent quality data.

			VFA (mg/L)				
Period	ClO ₄ ⁻ (mg/L)	COD (mg/L)	Acetic Acid	Propionic Acid	n-Butyric Acid	i-Butyric Acid	Valeric Acid
52-155	0.02 (±0.01)	14.10 (±7.50)	1.59 (±0.47)	-	-	-	-
156-318 333-384	0.01 (±0.01)	13.87 (±5.87)	0.03 (±0.13)	-	-	-	-
319-332	0.21 (±0.13)	11.00 (±3.61)	0.00 (±0.00)	-	-	-	-
385-532	0.00 (±0.00)	10.92 (±5.63)	1.01 (±1.54)	-	-	-	-

Table A2. Bioreactor-R effluent quality data.

			VFA (mg/L)				
Period	RDX (mg/L)	COD (mg/L)	Acetic Acid	Propionic Acid	n-Butyric Acid	i-Butyric Acid	Valeric Acid
52-155	0.20 (±0.15)	87.86 (±14.62)	41.69 (±3.85)	27.00 (±3.39)	-	-	-
156-231	0.17 (±0.06)	42.10 (±19.10)	19.67 (±5.28)	9.06 (±3.71)	-	-	-
232-384	0.13 (±0.22)	61.95 (±25.92)	37.94 (±17.25)	16.26 (±9.66)	-	-	-
385-532	0.00 (±0.00)	14.0 (±7.70)	4.24 (±2.10)	1.25 (±0.65)	-	-	-

Table A3. Bioreactor-P&R effluent quality data.

Period	ClO ₄ ⁻ (mg/L)	RDX (mg/L)	VFA (mg/L)						
			COD (mg/L)	Acetic Acid	Propionic Acid	n-Butyric Acid	i-Butyric Acid	Pivalic Acid	Valeric Acid
52–155	0.07 (±0.04)	0.56 (±0.17)	108.27 (±25.0)	59.76 (±20.00)	16.28 (±6.76)	0.43 (±0.20)	-	25.00 (±0.00)	-
156–318	0.02 (±0.02)	0.43 (±0.18)	27.20 (±8.97)	12.29 (±3.67)	-	-	-	25.00 (±0.00)	-
319–384	0.00 (±0.00)	0.13 (±0.04)	14.88 (±7.85)	8.37 (±4.51)	0.34 (±0.44)	-	-	25.00 (±0.00)	-
385–414	0.00 (±0.00)	0.12 (±0.03)	19.67 (±0.58)	5.94 (±0.97)	1.69 (±0.50)	-	-	25.00 (±0.00)	-
415–447	0.00 (±0.00)	0.10 (±0.05)	32.67 (±3.79)	9.52 (±4.79)	4.26 (±3.18)	-	-	25.00 (±0.00)	-
448–471	0.00 (±0.00)	0.11 (±0.01)	30.50 (±4.95)	12.01 (±3.64)	5.82 (±1.52)	-	-	25.00 (±0.00)	-
472–590	0.00 (±0.00)	0.10 (±0.03)	22.67 (±12.2)	6.37 (±5.35)	2.87 (±2.83)	-	-	25.00 (±0.00)	-

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14. ABSTRACT <p>Perchlorate, an oxidizer routinely used in solid rocket motors, is easily washed out of old motors. The washout operation, however, leads to wastewater containing perchlorate. Royal Demolition Explosive (RDX), a major component of military high explosives, is transferred to Army industrial wastewaters during assembly of new munitions and during demilitarization of old munitions. New high explosives contain perchlorate, commingling perchlorate and RDX for the first time. In addition to their damaging effect to the environment, perchlorate and RDX can also be detrimental to human health.</p> <p>This study focused on determining the effectiveness of removing perchlorate and RDX individually and when commingled, using ethanol as an electron donor at steady-state conditions. Three laboratory-scale anaerobic fluidized bed reactors were monitored. A fourth reactor, located at McAlester Army Ammunition Plant, currently used to treat pinkwater, was also tested.</p> <p>The experimental results demonstrated that the biodegradation of perchlorate and RDX was more effective in bioreactors receiving a single contaminant than in the bioreactor fed both contaminants. Results from perturbation experiments revealed that competition within the microbial consortia for substrate played a major role in determining the structure of that community.</p>					
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